Characterising Distinct Mechanisms of Interleukin-12-mediated Tumour Suppression

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This work is dedicated to Lotta, Nene and Oscar.

Disclosure

The data depicted in figures 12 to 16 were acquired in a shared project with Kathrin Nussbaum and will also be part of her thesis. Together, we collaborated in planning, performing and interpreting of the described experiments. The figure legends will specify who conducted the experiments depicted in the corresponding figure.

Further, K. Nussbaum may use figures 17 and 18 in her thesis.

Experiments shown in the appendix are published in the following manuscript:

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Table of contents

I Summary

Treatment with immune modulating components is about to revolutionise cancer therapy. One promising candidate, demonstrating potent anti-tumour responses is the immune mediator interleukin-12 (IL-12). Various immune cell types have been implied in the IL-12-induced tumour rejection, but different studies have presented conflicting results. Our group previously described the essential role of innate lymphoid cells (ILCs), depending on the transcription factor RORγt, during the IL-12-mediated anti-tumour response. The co-transfer of only 10'000 RORγt dependent ILCs with IL-12 secreting tumour cells prevented tumour growth, while other immune cells were unable to elicit this effect.

In this thesis I investigated whether therapeutic delivery of IL-12 to established tumours, provoked a similar ILC-dependent anti-tumour response. We discovered that the late therapeutic treatment failed in the absence of interferon-γ (IFNγ), while early preventive administration was independent of this cytokine. Upon late IL-12 administration IFNγ was predominantly secreted by natural killer (NK) cells and T lymphocytes and the absence of the lymphoid compartment caused IL-12 treatment to fail. Notably, specific depletion of RORγt dependent ILCs, NK or T cells did, however, not influence IL-12-mediated tumour immunity. Thus, we excluded the requirement of those cells to respond to IFNγ in this context. IFNγ signalling led to the accumulation and activation of monocytes and their progeny within treated tumours. Nonetheless, these cells were shown to be dispensable for the anti-tumour response, as IL-12-treated tumours were suppressed in the absence of monocytes. Moreover, direct IFNγ signalling inhibited endothelial cell growth and induced the expression of adhesion molecules on tumour vessels. Endothelium specific ablation of IFNγ signalling, however, had no impact on the tumour suppression upon late treatment with IL-12. Thus, we have excluded the involvement of specific cell types that have previously been suggested to contribute to the IL-12 mediated anti-tumour immunity.

Furthermore, we aimed to characterise the phenotype and function of tumour suppressive ILCs upon early preventive treatment with IL-12. Whereas the transfer of spleen-derived RORγt-dependent ILCs potently rejected solid tumours, gut-derived ILCs failed to do so. A comparison of RORγt-dependent ILCs in spleen and gut showed differences in their phenotype and transcriptional profile. In fact, splenic ILCs had down-regulated RORγt expression, upregulated the transcription factor T-bet and phenotypically resembled NK cells. Moreover, they were shown to be responsive to IL-12 and secreted IFNγ upon stimulation. In contrast, gut-derived ILCs expressed lower levels of the IL-12 receptor and retained RORγt expression. These differences in IL-12 responsiveness of ILCs, may explain the distinct functions of splenic and gut ILCs, regarding the IL-12-mediated tumour suppression.

II Zusammenfassung

Die medikamentöse Regulation des Immunsystems hat der Therapie von bösartigen Tumoren erhebliche Fortschritte gebracht. Eine der vielversprechenden Substanzen, ist der Immun-Botenstoff Interleukin-12 (IL-12), der das Wachstum verschiedener Tumore hemmt. Diese Tumorsuppression wurde mit der Wirkung von IL-12 auf diverse Zellen des Immunsystems in Verbindung gebracht, wobei unterschiedliche Studien widersprüchliche Resultate präsentierten. Unsere Forschungsgruppe konnte zuvor eine zentrale Rolle von ILCs (innate lymphoid cells), deren Entwicklung vom Transkriptionsfaktor RORγt abhängt, in der IL-12-induzierte Hemmung des Tumorwachstums beschreiben. Der Ko-Transfer von nur 10'000 RORγt abhängigen ILCs, zusammen mit IL-12 produzierenden Tumorzellen, vermochte die Zunahme der Tumormasse zu verhindern.

In dieser Arbeit habe ich den therapeutischen Effekt von IL-12 auf etablierte Tumore mit dem einer präventiven Verabreichung verglichen. Während die Immunantwort nach therapeutischer IL-12 Injektion Interferon-γ (IFNγ) benötigte beruhte der Erfolg der präventiven Verabreichung nicht auf diesem Botenstoff. Die Injektion von etablierten Tumoren mit IL-12 stimulierte die Sekretion von IFNγ durch NK (natural killer) und T Zellen und in der Abwesenheit von Lymphozyten versagte die IL-12 Therapie. Die spezifische Depletion von RORγt abhängigen ILCs, NK und T Zellen hatte jedoch keine Auswirkungen auf den therapeutischen Effekt von IL-12, weshalb wir die Notwendigkeit einer Stimulation dieser Zellen durch IFNγ ausschlossen. IFNγ führte zur Akkumulation und Aktivierung von Monozyten und deren Nachkommen im Tumorgewebe. Die IL-12-induzierte Anti-Tumorantwort wurde durch die Depletion von Monozyten nicht beeinflusst, weshalb auch die Rolle dieses Zelltyps vernachlässigbar war. Zusätzlich hemmte IFNγ das Wachstum von Endothelzellen und steigerte die Expression von Adhäsionsmolekülen auf Blutgefässen. Diese Effekte konnten durch die spezifische Entfernung des IFNγ Rezeptors auf Endothelzellen neutralisiert werden, was jedoch keinen Einfluss auf die Tumorsuppression durch IL-12 hatte. Somit konnten wir die Beteiligung von Zelltypen, deren Funktion während der Anti-Tumoraktivität von IL-12 in der Literatur häufig besprochen wurde, ausschliessen.

Im Weiteren widmeten wir uns in der folgenden Arbeit der Charakterisierung von tumor-hemmenden ILCs nach präventiver Verabreichung von IL-12. Dabei wurden Funktion und Phänotyp von RORγt abhängigen ILCs aus Milz und Darm verglichen. Während der Transfer von ILCs aus der Milz das Tumorwachstum verhinderte, konnte die Injektion von Darm ILCs diesen Effekt nicht reproduzieren. Milz ILCs hatten die Expression von RORγt eingestellt und exprimierten anstelle dessen den Transkriptionsfaktor T-bet und NK Zell charakteristische Oberflächenmoleküle. Im Gegensatz zu Darm ILCs, welche die Expression von RORγt beibehielten, konnten wir in Milz ILCs eine erhöhte Transkription des IL-12 Rezeptors feststellen. Die ungleiche Reaktionsfähigkeit auf IL-12, könnte die Unterschiede von Milz und Darm ILCs bezüglich Anti-Tumor-Antwort, erklären.

III Frequently used abbreviations

APC	antigen presenting cell
ATP	adenosine 5’-triphosphate
B16F10-IL-12Fc	B16F10 cells secreting IL-12Fc
CD	cluster of differentiation
CCR2	chemokine receptor 2
Ccr2$^{-/-}$	mice deficient for the chemokine receptor CCR2
cDC	conventional DCs
Cre	loxP site-specific recombinase
CTLA-4	cytotoxic T lymphocyte antigen-4
DAPI	4',6-diamidino-2-phenylindole dilactate
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ECM	extracellular matrix
eGFP	enhanced green fluorescent protein
EMT	epithelial-mesenchymal transition
Eomes	eomesodermin
eYFP	enhanced yellow fluorescent protein
FCS	fetal calve serum
FDA	food and drug administration (US)
GM-CSF	granulocyte-macrophage colony-stimulating factor
HBSS	hank’s balanced salt solution
Id2	inhibitor of DNA binding 2
ICAM-1	intercellular adhesion molecule-1
IFN	interferon
Ifngr1$^{-/-}$	IFNγ receptor deficient mice
Ifngr$^{fl/fl}$	conditional knock out mice for IFNγ receptor
IL	Interleukin
IL12rb2$^{-/-}$	IL-12 receptor β2-deficient mice
Il15ra$^{-/-}$	IL-15 receptor-deficient animals
ILC	innate lymphoid cells
iNOS	inducible nitric oxide synthase
i.t.	intratumoural
i.v.	intravenous
Jak	Janus kinase
loxP	recognition sequence for Cre
LP gut	lamina propria from the small intestines
LTi	lymphoid tissue inducer cell
LysM	Lysozyme 2
MAPK	mitogen-activated protein kinase
MCA	3’-methylcholanthrene
MDSC	myeloid-derived suppressor cells
MHC	major histocompatibility complex
MHCII^{+}	MHC class II expressing cell
MMP	matrix metalloprotease
mRNA	messenger ribonucleic acid
MS-1	MILE SVEN 1 endothelial cell line
NCR	natural cytotoxicity receptor

NET	neutrophil extracellular trap
NK cell	Natural killer cell
NKG2D	natural killer group 2 member D
NKT cell	natural killer T cell
NO	nitric oxide
pDC	plasmacytoid DCs
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD-L1	programmed death-ligand 1
PRR	pattern recognition receptor
pSTAT	phosphorylated STAT
R	receptor
RAG	recombination activating gene
Rag2$^{-/-}$ *Il2rgc*$^{-/-}$	mice deficient for the common gamma chain of the IL-2 receptor and RAG2
Rag1/2$^{-/-}$	RAG1/2-deficient mice
Rb	retinoblastomaprotein
Rorc$^{-/-}$	RORc deficient mice
Rorc^{fm+}	Rorc-Cre x ROSA26-stop$^{fl/fl}$-eYFP fate map mice
Rorc^{rep+}	RORc-eGFP reporter mice
RORγt	retinoic acid related orphan receptor γt
RORγt^{fm+}	YFP expressing RORγt dependent cells from Rorcfm+ mice
RORγt^{rep+}	GFP and RORγt expressing cells from Rorcrep+ mice
ROS	reactive oxygen species
s.c.	subcutaneous
SPLC	splenocytes
STAT	signal transducer and activator of transcription
TAM	tumour-associated macrophages
Taq	polymerase from Thermus aquaticus
TCR	T cell receptor
TGF-β	transforming growth factor-β
TH cell	T helper cell
TNF	tumour necrosis factor
Treg	regulatory T cell
UV	ultraviolet
VCAM-1	vascular cell adhesion molecule-1
VEC	VE-Cadherin
VEGF	vascular endothelial growth factors
WT	wild type

IV Introduction

» 1 Cancer

For a long time, cancer was considered to be a burden of the developed world. This resulted from the improved control over infectious diseases, causing increased life expectancy, which was accompanied by a higher incidence of malignancies. In 1970, only 15% of cancers were diagnosed in developing countries. This frequency, however, rose up to 56% in 2008, highlighting the global dimension of the disease. Today, the risk of contracting cancer is increasing worldwide, which reflects the growing population, changes in age distribution and exposure to risk factors (1). While the incidence is still considerably elevated in high- compared to low-income countries, differences in mortality rates are less pronounced (1). This clearly illustrates the lack of accessibility to cancer therapeutics in developing countries. In high-income countries, therapeutic assessment rarely leads to the cure of cancer, but more frequently prolongs survival (2). This implies a high prevalence of cancer and a large number of patients that are under constant treatment. It is obvious that the costly therapies create a socio-economic problem, which can be expected to grow in concordance with cancer incidence.

Thus, there is a desperate need for novel effective therapeutic strategies that can be afforded by low-income countries. Development of such agents in turn requires in depth knowledge of the mechanisms, by which cancers develop characteristics of malignancy. For this reason, the authors Hanahan and Weinberg defined a set of hallmark capabilities, which cancer cells need to acquire, in order to successfully evolve (3). These capabilities are attained by mutations in a multistep process, which has similarities to Darwinian evolution. Driving mutations lead to the gain of function of oncogenes or loss of function of tumour suppressor genes and finally convert a healthy cell to a cancer cell. More recently, the authors revised their initial concept, by adding further hallmarks to the development of cancer (4). Partially, this was motivated by the increased awareness that tumours, rather than merely consisting of cancer cells, involved a plethora of adjacent stromal cells, actively contributing to growth and invasiveness. Together, these cells form the “tumour microenvironment”, among others comprising endothelial cells, immune cells and tumour-associated fibroblasts. The following section will briefly summarise the hallmarks of cancer as defined by Hanahan and Weinberg, which will be illustrated by examples of frequent mutations in human melanomas.

»1.1 The hallmarks of cancer

»1.1.1 Sustaining proliferative signalling

In healthy cells, proliferation is tightly regulated to preserve the specific architecture and function of tissues. The cell only enters the growth and division cycle upon stimulation with growth factors. These growth factors are either components of the surrounding extracellular matrix (ECM) or secreted by neighbouring cells in a paracrine manner. Likewise, systemically released hormones can induce proliferation by binding to correspondent receptors. Cancer cells acquire mutations that allow them to circumvent this growth control by the environment. Some tumours adapt by secreting their own growth signals, resulting in autocrine growth stimulation. Moreover, changes of the signalling receptor, such as increased expression, structural alterations or modification of receptor composition, may lead to hyper responsiveness to proliferative signals. This is for instance observed in advanced melanoma, which over-express the epidermal growth factor receptor (EGFR) (5,6). Alternatively, mutations in the signalling pathway downstream of growth receptors can lead to constitutive activation of the growth cycle. For instance, activation of the RAS-RAF-MEK-ERK-mitogen-activated protein kinase (MAPK) pathway is observed in various types of cancer, including melanoma. BRAF and NRAS mutations are frequently detected in melanomas and result in the activation of the MAPK in absence of extracellular stimuli (7,8).

»1.1.2 Evading growth suppressors

In addition to proliferation promoting molecules, there are signals that inhibit cell division. Similar to growth factors, these molecules are secreted as soluble growth inhibitors, immobilised in the ECM or expressed on the surface of neighbouring cells. These inhibitory signals can interrupt mitosis, leading to temporary cell quiescence or inducing cell differentiation and permanent growth suppression. Avoidance of such anti-proliferative signals supports the inordinate growth of tumours. Nearly all anti-mitotic signals are managed by the tumour suppressors retinoblastoma protein (Rb) and p53 (4). While Rb mainly responds to extracellular signals, p53 blocks proliferation upon intra-cellular stress, such as DNA damage and lack of nutrients or oxygen. Mutations leading to a loss of function of Rb or p53 are frequently observed in cancer. Genetic alterations of these pathways are also common in melanomas arising in familial settings (7). A third of hereditary melanomas harbour a germline mutation in the cyklin-dependent kinase inhibitor 2A (CDKN2A) locus (9). Loss of the functional CDKN2A gene leads to suppressed Rb and p53 activity and thereby unleashes the proliferative potential of cancer cells (9).

»1.1.3 Resisting cell death

Besides the regulation of proliferation and growth, cell death is an important process for organ development and tissue homeostasis. Under physiological conditions, cell death occurs in a highly programmed fashion, termed apoptosis (10). Induction of apoptosis relies on the presence of pro-apoptotic extracellular ligands, the loss of anti-apoptotic survival signals or intracellular signs of cell damage. Since these signals are abundant in cancer cells (4), blocking pathways involved in the induction of apoptosis is beneficial for the tumour. Most of these pathways result in the release of mitochondrial cytochrome C and subsequent activation of caspases, initiating the cascade of defined steps characteristic for apoptosis (10). The p53 tumour suppressor can, for instance, induce apoptosis by stimulating cytochrome C release. Moreover, the expression of pro- and anti-apoptotic members of the B cell lymphoma 2 (Bcl-2) family, which control cytochrome C release, have been found to be deregulated in melanomas (11).

Cell death by necrosis is less organised and results in the loss of cell membrane integrity and discharge of intracellular components into the extracellular space. Thus, necrosis usually triggers an inflammatory response and infiltration of immune cells. Such immune cells can be exploited by tumour cells to further promote cell proliferation by providing growth signals (12). Thus, tumours may to a certain extent profit from cell death.

»1.1.4 Enabling replicative immortality

In addition to the deregulation of proliferation and cell death, cancer cells need to ensure limitless replication. Cell intrinsic pathways control the maximal number of division cycles that each cell is capable to undergo. After reaching this limit, cells enter the state of senescence, in which they fail to further propagate. If senescence can be circumvented, cells proceed to the phase of crisis, which in most cases will inevitably lead to chromosomal damage and cell death (13). Repetitive nucleotide sequences at the end of chromosomes called telomeres constrain replication. With each cell division, these telomeres will lose a number of base pairs and thereby erode. The DNA polymerase telomerase can actively insert nucleotides to the telomeres and thereby postpones senescence and crisis. Most cancer cells are immortalised by over-expressing telomerase (13). In melanomas telomerase activity has been correlated with a particularly poor prognosis and is indicative of tumour progression (14).

»1.1.5 Inducing angiogenesis

In developed healthy tissues, blood vessels are largely quiescent, apart from transient activation observed during wound healing and tissue remodelling. In contrast, growing

cancers require the sprouting of vasculature (angiogenesis) to maintain the supply of nutrients and oxygen, and the disposal of metabolic waste. Thus, tumours promote the release of pro-angiogenic factors and inhibit anti-angiogenic molecules, a phenomenon termed “angiogenic switch” (15). Pro-angiogenic factors can be secreted as soluble mediators, as in the case of vascular endothelial growth factors (VEGFs). In tumours, the expression of VEGFs is upregulated by both hypoxia and oncogene signalling (16). VEGFs can also be captured within the ECM and are liberated by proteases, such as the matrix metalloprotease-9 (MMP-9) (17). Immune cells attracted to the tumour tissue can further support angiogenesis by various mechanisms (18).

» 1.1.6 Activating invasion and metastasis

Many cancers develop the ability to invade tissues and form metastases, which is the emergence of tumour cells distant from the primary tumour. Cancer cells can thereby populate new niches, which offer more nutrients and space. For the host, this progression is less desirable, as metastases are the main cause of death in cancer patients (19). The formation of distant metastases involves evasion of the primary tumour to blood or lymph vessels, the invasion into a new microenvironment and adaptation to novel conditions (20). Initially, the cancer cell detaches from its surrounding by altering cell-to-cell and cell-to-matrix adhesion. Melanoma cells and a variety of invasive carcinoma cells, show an impaired attachment through the adhesion molecule E-cadherin (21,22). The expression of integrins that mediate cell-to-matrix contact has been shown to shift during the process of invasion (23). The release and activation of ECM degrading proteases by cancer cells or adjacent tissue, further promotes invasive capabilities (24). Many of these alterations are part of the epithelial-mesenchymal transition (EMT), a program that is important during migratory processes in embryonic development (25). Activation of the EMT leads to multiple cellular alterations that support the development of the cells invasive phenotype. Thus, it has been suggested that reactivation of EMT contributes to cancer progression (25). Due to the essential role of EMT during the dissemination of neural crest cells – which are the ancestors of melanocytes – it is not surprising that many EMT related genes are altered in melanomas (11,26).

» 1.1.7 Genome instability and mutation

The hallmarks described so far depend on alterations on the genomic level, which are achieved by mutations or epigenetic influences. As spontaneous mutations usually occur at a low frequency, cancer cells acquire the means to increase mutation rates. Healthy cells possess an entire DNA-maintenance-machinery that detects genomic damage. While minor

DNA damage is rapidly repaired, the cell enters senescence or undergoes apoptosis upon more extensive damage. Thus, alterations in this machinery can influence mutation rates. Whereas in hereditary cancers, mutations often affect DNA repair genes, these mutations are rare in sporadic cancer (27). In contrast, spontaneous tumours are thought to accumulate mutations due to oncogene-induced replication stress (27). The dysfunction of telomerases can lead to chromosomal damage, which contributes to genomic instability (28).

»1.1.8 Reprogramming energy metabolism

Cells generate energy by oxygen-independent glycolysis followed by oxidative phosphorylation in mitochondria. Both pathways serve to produce the high-energy molecule ATP (adenosine 5'-triphosphate), but oxidative phosphorylation yields around 20 times more ATP than anaerobic glycolysis. Nevertheless, cancer cells predominantly gain ATP by glycolysis even under normoxic conditions, a phenomenon which is known as Warburg effect (29). This is partly achieved by upregulating glucose transporters and is pronounced by the lack of oxygen (30). It is not completely understood, why cancer cells undergo this metabolic switch independently of tissue oxygenation. Due to abnormal architecture of the tumour vasculature, oxygenation may fluctuate, which implies an advantage for cells that rely on glycolysis. It has also been suggested that glycolytic intermediates may fuel the generation of nucleosides and amino acids, required for the rapid growth of cancer cells (31).

»1.1.9 Hallmarks involving the immune system

The last two hallmarks **"tumour-promoting inflammation"** and **"evading immune destruction"** involve the interplay of cancer cells with the immune system. These titles allude to the ambivalent role that immune cells have been attributed in the context of cancer. On the one hand, they have been described to recognise altered tumour cells and successfully eliminate them during different phases of tumour growth. On the other hand, the constant surveillance by the immune system exerts a selective pressure on the cancer, which in turn shapes the tumour landscape, a phenomenon that was termed cancer immunoediting (32). Furthermore, sustained inflammation can promote the development of cancer, and supports growth and establishment of invasive features of established tumours (12). These aspects of tumour immunity and the role of distinct cellular players will now be introduced in some more detail.

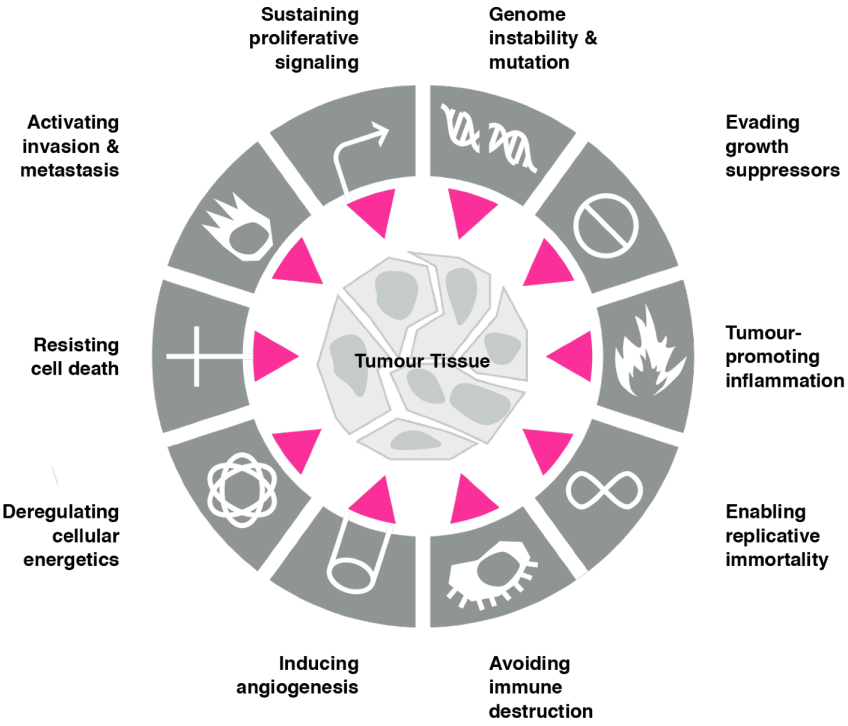


Illustration 1: The hallmarks of cancer. Illustration depicting the hallmark capabilities that cancer cells have to develop to ensure progressive growth and avoid destruction. These capabilities involve overcoming cell intrinsic hurdles such as limited growth signals, growth suppression, cell death upon damage, mechanisms of DNA-repair and limited cell proliferation. The ability to promote angiogenesis, to metastasise and to alter their metabolism, supplies the tumour with nutrients and oxygen, creates new niches and allows them to generate energy in a hypoxic environment, respectively. The immune system plays a dual role in carcinogenesis as it can both eliminate cancer cells and promote tumour development. These capabilities are supported by cells in the tumour microenvironment; here simplified in the tumour tissue. The Illustration is adapted from Hanahan and Weinberg, 2011 (4).

» 2 Cancer and the immune system

» 2.1 Immune cells involved in the detection of cancer

The interplay of the immune system and cancer implies that the immune cells can detect aberrant cancer tissue. While leukocytes recognise pathogens by structural patterns that are perceived as non-self, tumours may resemble healthy tissue. As a result, the immune system develops mechanisms of tolerance, which allow the cancer to grow unhindered. The theory that the leukocytes can respond to so-called “danger signals” may, however, explain the development of protective immune responses towards cancer (33). Tissue trauma or stress causes cell death, which leads to the release of danger signals, such as heat shock proteins (33). Binding to pattern recognition receptors (PRRs), danger signals and microbial patterns alert cells of the innate immune system, which form the first line of immune defence. While the response of innate immune cells is generic, due to their conserved receptors, cells of the adaptive immune system detect threats like pathogens or cancer cells more specifically. Their receptors can bind to a large variety of molecules, called antigens. Compared to the immediate action of innate cells the adaptive response needs several days to develop, as adaptive cells require the presentation of their cognate antigen by innate cells. Upon this interaction, adaptive cells are activated, clonally expand and elicit a potent immune response. Apart from the distinction of innate and adaptive immunity, developmental and morphological features are used to classify immune cells into the lymphoid and myeloid compartment. While lymphocytes are found within both the innate and adaptive arm of the immune system, myeloid cells comprise most of the innate compartment.

» 2.1.1 The myeloid compartment

This group of cells comprises granulocytes, monocytes, macrophages and dendritic cells (DCs). After birth, these cells derive from myeloid progenitor cells in the bone marrow (34). **Monocytes**, expressing the surface markers CD11b and F4/80, circulate in the blood and can further give rise to macrophages and DCs. This differentiation is particularly pronounced during inflammatory responses, which lead to the recruitment of $Ly6C^+$ inflammatory monocytes through chemokine receptor 2 (CCR2) signalling (35).

A common feature of myeloid cells is the ability to ingest macromolecules (phagocytosis), such as pathogens or cellular debris, upon recognition. Phagocytosis is primarily the function of tissue macrophages and **neutrophil granulocytes**, the latter expressing the marker Ly6G. Upon phagocytosis, the ingested macromolecules are destroyed by proteolytic enzymes, reactive oxygen species (ROS) or anti-microbial proteins, which can be stored in

granules. Apart from this intracellular killing mechanism neutrophil granulocytes can also excrete such substances to the extracellular space, achieved by the process of degranulation (36). More recently they have been shown to release fibers, forming web-like structures called neutrophil extracellular traps (NETs), which are mainly composed of DNA. Microorganisms captured within those traps are prevented from spreading and killed by antimicrobial substances and proteases attached to the NETs (37). Neutrophil granulocytes are also involved in healing processes by removing cell debris, reorganising the ECM and promoting revascularisation. The latter two features are, for instance, achieved by the secretion of the metalloproteinase MMP-9 (36).

In contrast to the short-lived neutrophil granulocytes **macrophages** can reside in tissues for a long time. Apart from developing from monocytes and stem cells in the bone marrow, a number of macrophages were demonstrated to originate from embryonic yolk sac precursors (38). These yolk sac-derived macrophages populate tissues like skin, lung, spleen and kidney and are thought to self-renew locally. Such cells can be separated from bone marrow-derived macrophages by their elevated expression of F4/80 and low levels of CD11b on their cells surface (39). Among other myeloid cells, macrophages are potent producers of immune signalling molecules called cytokines. Upon activation through PRRs, macrophages, for instance, secrete the pro-inflammatory cytokines Interleukin-1β (IL-1β), tumour necrosis factor (TNF), and IL-12 (40). Such cytokines can provoke an inflammatory response and direct the subsequent adaptive immunity.

Myeloid subsets can present exogenous antigens on their surface, which they obtain from the degradation of ingested macromolecules. Cells with such function are referred to as antigen presenting cells (APCs). APCs express major histocompatibility complex (MHC) class I and II molecules, by which they present peptide antigens to the adaptive αβ T cells in lymphoid tissues. In combination with co-stimulatory factors and cytokine signalling this interplay of APCs with lymphocytes will lead to the activation of adaptive immune responses (41). While MHC class II binds exogenous peptides, MHC class I molecules mainly present intracellular antigens. In fact, all nucleated cells express endogenous peptides bound to MHC class I on their surface, which is how cytotoxic T cells can detect foreign antigens on infected or aberrant cells. In contrast to other somatic cells, specialised APCs can, however, express extracellular peptides on MHC class I. This process is known as cross-presentation and implies that the APC itself is not required to be infected or malignantly transformed to present antigens on MHC class I molecules (42).

DCs have been shown to be the most potent antigen presenting cells (43). To date this group of cells is sub-divided into plasmacytoid (pDCs) and conventional (cDCs) DCs. The first

subset is primarily detected in blood and lymphoid organs and secrete large amounts of the type I interferons (IFNs). In contrast, cDCs populate non-lymphoid and lymphoid tissues. In non-lymphoid organs a population of $CD103^+CD11b^-$ cDCs can be distinguished from $CD11b^+$ cDCs. A proportion of $CD11b^+$ cDCs develop from circulating monocytes. Non-lymphoid cDCs are highly migratory and can also be found in lymphoid tissues. Further, lymphoid organs harbour $CD8^+CD11b^-$ and $CD8^-CD11b^+$ cDCs. All DC subsets are characterised by variable expression of CD11c. This surface marker is, however, not specific for DCs, as it is also expressed on other cells, such as macrophages (44).

»2.1.2 The lymphoid compartment

Cells within this compartment arise from common lymphoid progenitor cells in the bone marrow and share the morphology of a small cell with a round nucleus and scarce cytoplasm. The lymphoid compartment comprises natural killer (NK) cells, T cells, NKT cells and B cells.

NK cells do not directly target pathogens, but detect and eliminate infected or aberrant cells. In order to recognise a compromised cell, NK cells express a set of activating and inhibitory surface receptors. The balance between signalling through those receptors regulates the activation status of the NK cells (45). Activation can occur upon binding of surface molecules that are induced on target cells by cellular stress. The activating receptor natural killer group 2 member D (NKG2D), for example, detects various ligands that are induced upon DNA damage (46). NK cell function is further sustained by cytokine signalling; in particular IL-15 and IL-12 are known to be strong stimulators (47). IL-15 is further required for NK cell maturation as shown by the paucity of NK cells present in IL-15 signalling-deficient mice (48). Inhibitory signals can be provided by receptors that recognise MHC class I molecules on the surface of target cells. The loss of MHC class I expression is often observed on cancer cells, which renders them susceptible to NK cell killing (46). Upon activation, NK cells exert effector functions, such as the release of perforin and granzyme, which induce apoptosis of the target cell. Furthermore, stimulated NK cells secrete cytokines, such as IFNγ. Due to their lack of antigen specificity and ability to elicit prompt responses, they are thought to belong to the innate arm of the immune system. The activating receptor NKp46 was described to be the most specific marker for NK cells of different species (49). This natural cytotoxicity receptor (NCR) was recently also detected on subsets of innate lymphoid cells (ILCs). NK cells are now thought to belong to a family of ILCs (50), which will be described later.

T cells and B cells recognise pathogens or transformed cells by specific receptors, which are generated by random somatic gene recombination (51). While each clone expresses one

antigen specific receptor, the diversity of genetically rearranged receptors within the population ensures that virtually any antigen is recognised. The receptor may also bind self-antigen, which is why the adaptive cells are subjected to a selection process, eliminating most self-reactive cells. Such cells elicit an effective primary immune response and subsequent encounter with antigen can provoke a more potent and more rapid immune response, a phenomenon that is ascribed to immunological memory (52). The development of T and B cells can be experimentally blocked by using mice deficient for the recombination activating genes (RAG) 1 and 2, which are required for the generation of antigen receptors (53,54).

Most T cells rearrange the α and β chain of the T cell receptor (TCR) locus. After their maturation and selection in the thymus they migrate to secondary lymphoid organs where they may encounter their cognate antigen presented by APCs. Upon binding to the peptide-MHC complex, TCR signalling initiates the stimulation of the T cell. T cells are characterised by the expression of CD3, a co-receptor associated with the TCR that is required for signal transduction. Upon T cell activation the expression of CD3 can be down-regulated (55), which may impede the detection of T cells *in vivo*.

$\alpha\beta$ T cells can be divided into a CD8 and CD4 expressing population. The function of **$CD8^+$ T cells** is central for immune responses against cancer cells. The TCR on $CD8^+$ T cells specifically binds to MHC class I presented peptides. Co-stimulatory signals and cytokines in addition to TCR engagement lead to the activation of $CD8^+$ T cells. Once they are primed, $CD8^+$ T cells can screen nucleated somatic cells for aberrant antigen bound to MHC class I molecules. Upon detection of their cognate antigen $CD8^+$ T cells can kill their target cell and are therefore called cytotoxic T cells. Similar to NK cells $CD8^+$ T cells can induce cell death upon the release of perforin and granzyme. Furthermore, CD8+ T cells express Fas ligand, which mediated apoptosis over the death receptor Fas on the target cell. Moreover, cytotoxic T cells secrete the cytokines TNF an IFNγ (reviewed in Andersen et al., 2006 (56).

$CD4^+$ T cells recognise peptides presented on MHC class II molecules. Their main function is to secrete a set of cytokines, which influence the subsequent immune response. Moreover, they support B cell function and cytotoxic T cell responses, and are thus referred to as T helper (T_H) cells. Depending on the composition of cytokines contributed by the APC, activated T_H cells can adopt distinct functions (57). T_H1 cells secrete IFNγ and IL-2 upon the up-regulation of the transcription factor T-bet. This subtype is known to support cytotoxic effector T cell functions. In contrast, T_H2 cells express the transcription factor GATA3 and secrete IL-4, IL-5, IL-10 and IL-13. A subset of T_H17 cells expresses the transcription factor retinoic acid related orphan receptor γt (RORγt) and secretes IL-17 (57). Although T_H

subtypes were thought to be terminally differentiated cells, it is now recognised that these cells are functionally plastic (58). Another T_H subtype has regulatory function as it establishes tolerance towards self and non-self antigens and is therefore termed regulatory T cell (Treg). Their development depends on the expression of transcription factor forkhead box protein 3 (FoxP3) and they secrete the immune suppressive cytokines IL-10 and transforming growth factor-β (TGF-β). Tregs can inhibit T cell activation by their expression of the cytotoxic T lymphocyte antigen-4 (CTLA-4), which binds to the co-stimulatory molecules CD80/86 on APCs (59).

A small fraction of T cells rearrange the γ and δ chain of the TCR locus. These γδ TCRs have a more limited diversity than αβ TCRs and are not restricted to MHC molecules. The scope of the antigens that **γδ T cells** respond to is not fully understood. After their maturation in the thymus, γδ T cells do not migrate to lymphoid organs but rather populate epithelial sites. Their response has innate-like kinetics and they can react to cytokines without further antigen stimulation. Therefore, γδ T cells are considered to have functional properties that can be placed in between innate and adaptive immunity (reviewed by Vantourout and Hayday, 2013 (60)).

Also **NKT cells** act at the interface of adaptive and innate immune responses. These cells express a semi-invariant αβ TCR that recognises lipid antigens presented on CD1d, a MHC class I-like molecule. In addition NKT cells express functional NK cell receptors and can produce T_H1- and T_H2-associated cytokines (61).

The role of **B cells** is generally neglected in studies of anti-tumour immune responses. Similar to T cells, B cells express rearranged B cell receptors (BCR), which is the membrane bound form of an antibody. These antibodies are secreted in a soluble form and are not limited to peptide recognition as are TCRs, but bind to a variety of chemical structures. Although tumour specific antibodies have been described and B cells frequently infiltrate tumour tissue, the induced responses have not been observed to be efficient (62). The ability of B cells to present antigens to T cells has rather been associated with a tumour-promoting role (63).

» 2.2 Cancer immunoediting

The idea that immune responses may influence the growth of cancer was motivated by the observation of cancer remissions upon infectious diseases (64). It was hypothesised that the immune system can eliminate cancer cells, limiting the number of clinically apparent malignancies, a phenomenon that was later termed “cancer immunosurveillance” (65,66).

Experimental evidence was contributed by the study of immune-deficient rodents. Mice, lacking either cellular components or molecular players of the immune system, were shown to be more susceptible to sarcoma formation, upon induction with the carcinogen 3'-methylcholanthrene (MCA), compared to immunocompetent mice (67-69). The subsequent study by Shankaran and colleagues provided a new twist to the understanding of cancer immunity (32). MCA-induced tumours derived from immunocompromised mice, lacking adaptive immune cells, were spontaneously rejected when transplanted into syngeneic immunocompetent mice. In contrast, tumour transplantation from wild type donors to immunocompetent hosts, led to progressive growth of the cancer. This suggested that cancers, growing in immune-deficient mice, are more susceptible to immune responses and, thus, are more immunogenic. Besides surveilling tissues for tumour cells, the immune system also seems to shape the tumour quality, a phenomenon that was termed "cancer immunoediting" (32). By now cancer immunoediting is described as the interplay between the immune system and the cancer tissue, which comprises the three phases of elimination, equilibrium and escape (70-72).

»2.2.1 Elimination

This phase describes the phenomenon of immune surveillance. Elimination has never been visualised *in vivo*, but has rather been demonstrated to take place in an indirect way. The increased susceptibility of immunocompromised mice to develop carcinogen-induced, spontaneously arising and mutation driven cancers, indicated that the immune system prevented the occurrence of malignancies. Utilising mice deficient for distinct populations of the immune system suggested that most immune cell types took part in processes of the elimination phase (reviewed in Vesely et al., 2011 (72)). The involvement of different leukocyte populations seems to depend on the type of cancer, method of cancer induction, anatomic location or tumour growth rates (71,72).

First, the innate arm of immunity reacts to developing tumours via danger signals and damage-associated molecular patterns (DAMPs), released by the tumour itself or the damaged adjacent tissue (71). Moreover, stress ligands expressed on the cancer cells' surface may bind to activating receptors on NK cells, NKT cells, $\gamma\delta$ T cells and $CD8^+$ $\alpha\beta$ T cells. For instance, NKG2D-deficient mice have been shown to develop more mutation driven lymphomas and prostate cancer (73). The cytokine secretion by such cells further supports subsequent immunity. Especially IFNγ was attributed a major role in cancer elimination (67,74). Among other effects, IFNγ induces cytotoxic effector functions by NK and $CD8^+$ T cells, such as the release of perforin, a cytolytic protein that was shown to be essential in immune surveillance of cancer (69,75). In spite of this effective initial recognition and attack

of the cancer cells by innate immune cells, most systems were demonstrated to rely on the action of adaptive immune cells towards the tumour (71). These adaptive responses require the presentation of tumour antigens, which are distinguished by their high or low specificity (76). The expression of viral peptides, mutated proteins and cancer-germline antigens is highly specific for the tumour tissue and result in strong T cell activation. In contrast, low-specificity tumour antigens derive from the cancers tissue of origin or from over-expressed proteins. Such antigens normally induce immunologic tolerance, but have also been shown to elicit anti-tumour T cell responses (76).

» 2.2.2 Equilibrium

During equilibrium, the cancer and the immune system reach a state of dynamic balance. Although effector functions of the immune cells restrict tumour growth, they fail to eliminate neoplastic cells, which can be explained by the cancer cell heterogeneity. This phase is characterised by the cancer cells acquiring immune evasive mutations, while immune cells adapt to these alterations and is terminated by elimination or escape of the tumour. The phase of equilibrium may explain the long latency from the initial mutations that create the aberrant cancer cells, to the clinical appearance of the malignant neoplasia. Moreover, certain human cancers, such as melanoma and breast cancer, are renown for their potential to form distant metastases long after detection and successful treatment of the primary tumour. This cancer dormancy could be attributed to the processes of equilibrium (reviewed in Vesely et al., 2011 (72)).

Equilibrium was experimentally assessed by Koebel and colleagues who induced occult sarcomas by exposing mice to low doses of the carcinogen MCA (77). Subsequent depletion of $CD4^+$ and $CD8^+$ T cells, IFNγ or IL-12 yielded the outgrowth of such occult tumours, underlining the essential role of adaptive immunity for the phase of equilibrium. Notably, depletion of NK cells did not accelerate tumour growth (77). In a model of murine leukaemia up-regulation of programmed death-ligand 1 (PD-L1) on dormant tumour cells was found to inhibit the effector function of $CD8^+$ T cells (78) or even induced T cell apoptosis (79), demonstrating the progressive immune evasion of the cancer cells. Moreover, occult metastases were found early after formation of mutation-driven melanoma and the depletion of $CD8^+$ T cells resulted in progressive growth (80).

» 2.2.3 Escape

This phase is represented by the failure of the immune system to control tumour growth, which leads to the development of a clinically apparent malignant disease. Escape of the tumour can occur primarily, if the immune system does not succeed to recognise the cancer

cells, or can be the result of the equilibrium phase. The remaining tumour cells have adapted to evade targeting by leukocytes, which results in progressive tumour growth (reviewed in Vesely et al. (72)). Cancer cells can for instance evade the immune system by inhibiting the direct recognition by lymphocytes, as achieved by loss of NKG2D ligands (81) or tumour specific antigens. Similarly, the tumour can accumulate defects in its antigen processing and presentation machinery (71), which impairs $CD8^+$ T cell killing. This can be observed when the cancer cells lose their responsiveness to IFNγ, a cytokine affecting many components of the antigen presentation pathways (82). There is also evidence that cancers impair DC maturation and thereby hamper antigen presentation to T cells (83). In addition, many tumour cells evade cytotoxic immune functions by using their “resisting cell death” hallmark. For example, this is achieved by mutations of the death receptors for tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) (84) and Fas ligand (85).

Many of the qualities that allow the tumour to evade immune destruction are directed towards creating an immunosuppressive tumour microenvironment. Interestingly, this cancer-related immune suppression was observed to be local, as splenic T cells respond normally to administered antigen (86). This local effect is supported by the presence of immunosuppressive cytokines at the tumour site. Cancer cells can secrete TGF-β or stimulate its production by cells in the tumour microenvironment (87). The effects of TGF-β are versatile and mainly lead to the inhibition of DC, T cell and NK cell function (87). Moreover, IL-10 has primarily been attributed to a strong immune suppressive role in cancers with multiple targets (88). In patients, both TGF-β and IL-10 are correlated with poor prognosis or metastatic disease (88). Interestingly, TGF-β in the tumour tissue was shown to convert effector T cells into Tregs (89), which further contributes to the increased IL-10 and TGF-β levels and suppression of T cell activity. Tregs have also been suggested to accumulate at the tumour site due to local proliferation or enhanced migration (90). The latter mechanism is supported by increased chemokine expression (91). In fact, there is an array of chemokines derived from tumour cells or the surrounding stroma that support the migration of leukocytes to the tumour (92). Some immune cell types may, however, harm the cancer, which is why many tumours actively suppress leukocyte trafficking. Partly, this is achieved by down-regulating adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), on the tumour vasculature (93).

The interplay of cancer cells with leukocytes of the myeloid lineage has proven to be particularly profitable for tumour escape. The cancer is thought to suppress maturation of such cells, causing myeloid cells to display diverse immune suppressing functions. Due to their regulatory capacity, a group of immature myeloid cells found in tumour tissue has been

termed myeloid-derived suppressor cells (MDSCs) (94). The IFNγ-induced up-regulation of the inducible nitric oxide synthase (iNOS) in MDSCs is thought to inhibit T cell survival and function (94). It is disputed whether those cells should be regarded as a distinct myeloid subtype or if their immune suppression merely represents a functional state. Huang and colleagues suggested that the chemokine CCL2 and the corresponding receptor CCR2 are essential for the recruitment of such suppressive myeloid cells. In the absence of CCR2 signalling a reduced tumour growth was observed, which was attributed to the loss of myeloid suppression (95).

CCR2 is also involved in the attraction of tumour-associated macrophages (TAM). Notably, recent studies suggest that the majority of TAMs derive from $CCR2^{+}$ Ly6C+ inflammatory monocytes (96-98). Depending on their activation status, macrophages have been suggested to adopt pro-inflammatory or immunosuppressive functions (99,100). The majority of TAMs were shown to promote tumour growth by multiple mechanisms (101). TAMs have, for instance, been demonstrated to secrete IL-10 and TGF-β (102). Moreover, myeloid cells, in particular macrophages, potently support tumour angiogenesis by secreting of VEGF or MMP-9 (18). There is also evidence that secretion and activation of metalloproteinases by TAMs promote tumour invasiveness and metastasis (17).

» 2.2.4 Inflammation-induced cancer

Apart from inhibiting and promoting tumour growth, immune responses can also induce *de novo* cancer formation. It is well documented that chronic particularly inflammation increases the risk of developing cancer. Such correlations have been found in pathogen-mediated inflammation, such as hepatitis virus-induced hepatocellular carcinoma, and autoimmune diseases, as observed in inflammatory bowel disease patients. The constant tissue damage, and regeneration or remodelling during chronic inflammatory responses may contribute to the pathogenesis of such cancers. For instance, the tissue is exposed to ROS produced by myeloid cells, which are actively mutagenic to surrounding cells. The mechanisms by which inflammation can induce tumour development are similar to the immune cell functions during immunoediting (reviewed in Grivennikov et al., 2010 (12)).

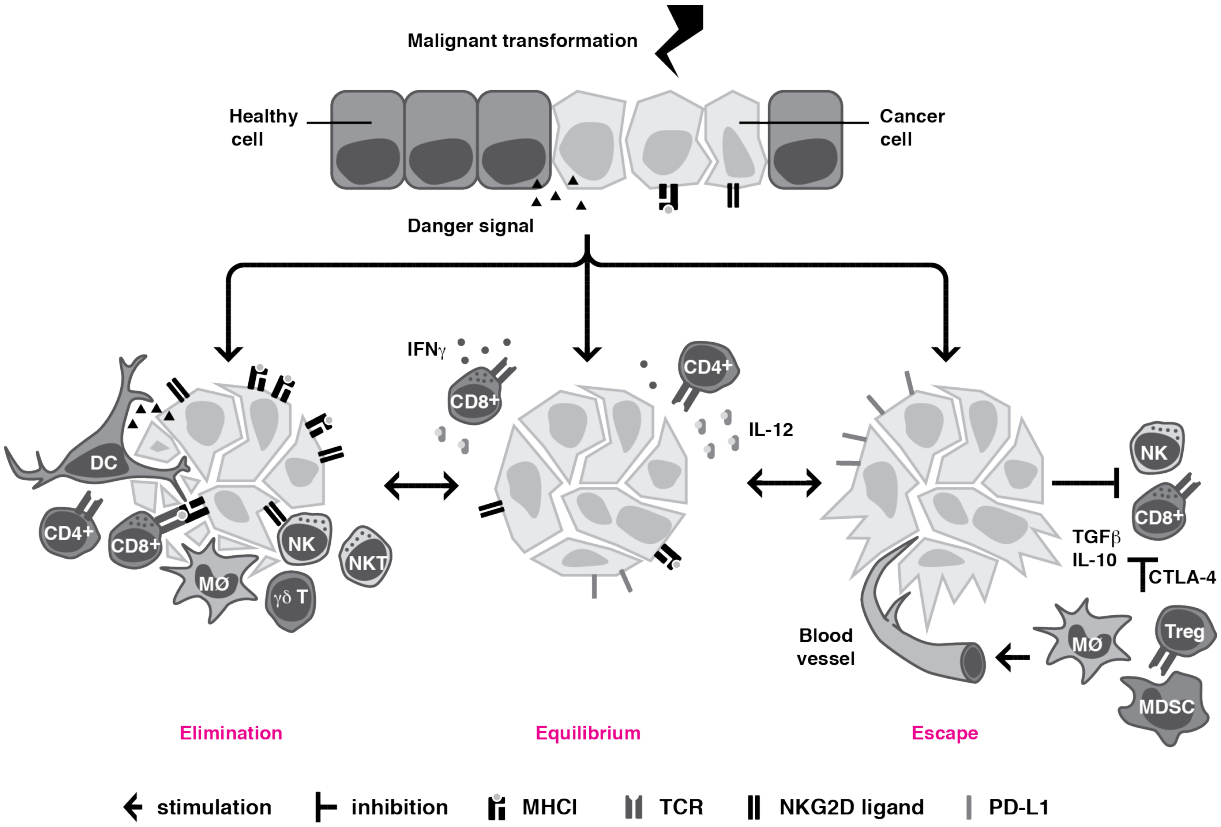


Illustration 2. The three phases of cancer immunoediting. Upon malignant transformation the cancer cells may succumb to anti-tumour immune responses (elimination), enter a dynamic balance with the immune system (equilibrium) or control and avoid intratumoural immune cells (escape). Highly immunogenic tumours present antigens and express immune stimulatory surface molecules, such as NKG2D leading to detection and elimination by leukocytes. These molecules are lost in poorly immunogenic cancers, which are dominated by the presence of immune inhibitory ligands, such as PD-L1. The selective pressure that the immune system exerts on the cancer ultimately supports tumour escape. In this phase cancer cells attract leukocytes with immunosuppressive function, fostering tumour invasiveness and angiogenesis. DC: dendritic cell, $CD4^+$: T helper cell, $CD8^+$: cytotoxic T cell, MØ: macrophage, γδ T: γδ T cell, NK: NK cell, NKT: NKT cell, MDSC: myeloid-derived suppressor cell, Treg: regulatory T cell. This illustration is adapted from Schreiber et al., 2011 (71).

»2.2.5 Evidence for cancer immunoediting in humans

Although the processes of immunoediting cannot be assessed experimentally in humans there is certain evidence that these take place in cancer patients. For instance, immune suppressed individuals generally have a greater risk of developing cancer (71). Patients suffering from acquired immune deficiency syndrome (AIDS) show an increased frequency of malignancies, although most of the cancers have a viral aetiology and are associated with herpes- or papilloma virus infection (103). The increase of virus-associated cancers also dominate reports, studying malignancies in recipients of organ transplants who are treated with immune suppressing drugs to avoid transplant rejection. A dramatic increase of non-melanoma skin cancer (200-fold) (104) and a more moderate elevation of melanoma cases have, however, been described in kidney transplanted patients (104,105). While these

findings demonstrate the failure of immunosurveillance, there is also data supporting the fact that the immune system may specifically respond to cancer cells and control tumour growth. The tumour infiltration with T, NK and NKT cells has been associated with improved prognosis in various malignancies (72). This link was first observed in melanomas (106,107) and was followed by the discovery of melanoma antigen specific T cells (108). The potent immune response elicited by antigen specific recognition is illustrated by the finding that such melanoma responsive T cells detect and attack healthy melanocytes upon treatment with immune modulating substances, leading to the clinical appearance of vitilligo (109). Remarkably, several case studies of spontaneously regressing melanomas were accompanied by the expansion of T cells (110-112).

» 2.2.6 Bright prospects for cancer immunotherapy

The potential of therapeutically promoting protective anti-tumour immunity and interfering with tumour escape spread hope for the treatment of cancer with immune modulating components, termed immunotherapy. The discovery of tumour antigens and responsive T cells that particularly brought vaccine based strategies into focus. The specificity of T cell responses to tumour tissue appears as an attractive target, as many conventional anti-cancer therapies are associated with strong side effects that result from damaging healthy tissues (113). Various strategies of vaccination are currently being clinically tested, and show promising results. Particularly melanoma patients have profited from this development, as some of the therapeutic approaches have shown curative effects (114). In the context of melanoma therapy, some of these treatment strategies will be further discussed in the next paragraph.

»3 Cutaneous melanoma

Cutaneous melanomas are highly malignant neoplasias arising from melanocytes. In humans, these cells predominantly reside within the basal layer of the epidermis and produce the pigment melanin. Characterised by their aggressive and invasive growth, melanomas can metastasise even at early stages of disease, when the tumour is small. Although they develop less frequently than non-melanoma skin cancers, melanomas are the main cause of skin cancer related death. While mortality rates have stabilised in Europe, the incidence of cutaneous melanomas is rising rapidly (7).

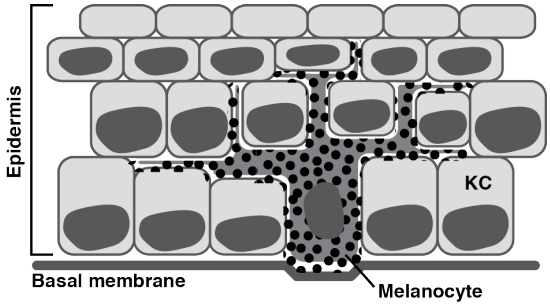


Illustration 3: Melanocyte residing in the skin. Illustration depicting a melanocyte resident within the basal layer of the human epidermis above the basal membrane. The keratinocytes (KC) forming the epidermis are provided with the pigment melanin, secreted by melanocytes.

»3.1 Development and Risk

Melanomas can arise form melanocytic naevi, benign lesions that are composed of melanocytes often referred to as moles. Therefore, individuals with large numbers of such naevi are at higher risk to develop melanomas (9,115). Naevi can appear to be morphologically atypical and are termed dysplastic naevi when fulfilling three of the following criteria: they exceed the size of 5 mm in diameter (I), show irregular colouring (II) or border (III), have an asymmetric contour (IV) or are accompanied by an erythema (V). These naevi in particular are correlated with an increased disease risk and should therefore be observed carefully (116). Only 20-40% of all melanomas, however, develop from naevi while 60-80% appear on healthy skin (117).

Ultraviolet (UV) radiation exposure has been causally linked to the incidence of cutaneous melanomas (118,119). Ultraviolet B radiation was recently shown to directly cause driving mutations, usually caused by C to T or G to A transitions, which supply melanoma cells with a fitness advantage (120,121). As pigments protect the skin against the impact of UV radiation, pale skin (phototype 1,2) predisposes individuals to developing melanomas. The

occurrence of melanomas in organs other than the skin, such as the gut and other mucosal sites demonstrates that melanomas can also establish independent of UV radiation.

Hereditary genetic conditions can increase the risk for developing melanoma. Some of these alleles predispose for a high risk, which is why several members of one family are affected by the disease, melanomas may occur in multiple locations and they arise early in life (9). Although such risk alleles are very rare they are characterised by a high penetrance and the resulting tumours account for up to 10% of all melanomas (122). Many of these mutations ultimately interfere with the Rb pathway or with the function of the tumour suppressor gene p53 (7). Low to intermediate risk alleles rather effect traits that render the individual susceptible to sporadic melanoma, such as fair skin (9).

»3.2 Diagnosis and Staging

Early visual recognition of melanoma is essential as it improves curative rates (7). Once a melanoma has been diagnosed, staging of the disease is performed to predict the individual prognosis and to define treatment strategies. A staging system has been introduced by the American Joint Committee on Cancer (AJCC) and is revised frequently (123,124). In the following paragraph this staging system will be briefly introduced.

Stage 0 melanomas are also called *in situ* melanomas and refer to the detection of aberrant melanocytes in the epidermal layer of the skin. The discovery of the cancer at this initial stage correlates with an excellent prognosis. Unfortunately, the minority of melanomas are diagnosed this early.

Stage I and II melanomas are established local tumours and are primarily evaluated by the tumour thickness, often termed Breslow's index, which predicts the survival rate of the patient accurately. The tumours are substaged using the thresholds of 1, 2 and 4 mm in thickness. This substaging is further fine tuned by using the ulceration status, as ulcerations usually predict an impaired prognosis. Moreover, assessment of primary mitotic rate of the cancer cells can complement the staging, as it was shown to be the second best predictor of survival rate after the tumour thickness at this stage.

Stage III melanomas have metastasised into local lymph nodes or into lymphatic vessels and are further substaged by the number of affected lymph nodes and the volume of the metastasis. Status of the sentinel lymph node involvement has been demonstrated to be the strongest overall prognostic factor in melanoma patients (124,125). Identification of lymph node metastases is mainly achieved by clinical detection, which can be complemented by sentinel lymph node biopsy (7,126). Also for this group tumour thickness and ulceration

status have been shown to well predict survival rates.

Stage IV melanomas are diagnosed when distant metastases have occurred. Metastases can arise virtually anywhere in the body but liver, lung, bones and brain are most often affected. The subcategories are defined by assessing serum levels for lactate dehydrogenase and the localisation of metastases. Regardless of the substaging stage IV disease is, however, hard to control and prognosis is poor (124).

»3.3 Treatment strategies

Melanomas have proven to be resistant to many conventional anticancer treatments, such as chemotherapy and radiation therapy. To date surgical excision of the primary tumour is the best treatment for this cancer type. Recently, increased understanding of the molecular biology of melanomas has led to the introduction of novel targeted therapies and immunotherapies. Many of these new compounds have shown to improve survival rates significantly and may revolutionise the treatment of melanomas (126). Unfortunately, virtually all treatments provoke side effects, few patients respond to therapy and cancers develop drug resistance. Thus, the combination of different treatment strategies is particularly attractive (7).

»3.3.1 Local therapy

Surgical assessment of melanomas is the standard treatment of the localised disease and involves wide local excision of the primary tumour. Depending on tumour thickness the recommended margin of resection will be adapted. For stage III patients with clinical involvement of the regional lymph nodes complete lymphadenectomy of the local lymphatic basin is performed. For metastatic patients at stage IV surgical treatment has limited benefits and is only implied as palliative treatment, when it has the prospects of increasing life quality (126,127). With the novel targeted and immune modulating drugs applied as pre-treatment before surgical intervention, resection of residual tumours at stage IV may become more attractive (126).

Radiation therapy is currently not routinely used in melanoma treatment as this form of cancer is considered to be relatively radiation resistant. It is usually considered for the adjuvant therapy of high-risk postoperative patients with stage III disease or melanomas localised on head or neck, for which surgical possibilities are limited (128). Moreover, stage IV patients with brain and bone metastases were shown to benefit from local radiation therapy (129). Although in this palliative setting, radiation therapy rarely increases the

survival of patients, it can improve their life quality by reducing symptoms (129).

»3.3.2 Systemic therapy

Systemic treatment is indicated at metastatic stages of melanoma disease. Until recently, chemotherapy with dacarbazin and immunotherapy with Interleukin-2 (IL-2) or IFNα were the only treatment options approved by the US food and drug administration (FDA) (130). In the last decade, however, major treatment advances have been achieved by the approval of monoclonal antibodies blocking CTLA-4 and BRAF kinase inhibitors (126). The molecular basis and effect of compounds currently used to treat melanoma patients are discussed in the following paragraph. Many more components and strategies are under investigation in pre-clinical and clinical trials.

Dacarbazin is an alkylating agent, which acts by attaching an alkyl group to the guanine base of DNA and thereby causes DNA damage and cell death. As cancer cells proliferate at higher frequencies compared to healthy cells, they are more susceptible to dacarbazin-induced DNA damage (130). Although, overall survival was never shown to be improved by dacarbazin, response rates are estimated at 10-30% (131). Treatment with dacarbazin also damages other proliferating tissues, leading to a variety of adverse effects. Damage to the gut mucosa causes nausea and vomiting, and impaired hematopoiesis results in leukopenia and anemia (130).

The term "**targeted therapy**" defines treatments using components that interfere with mutationally altered molecular pathways. As such genetic alterations can vary between melanomas, characterisation of the mutational landscape previous to treatment is essential. In melanoma therapy, targeted agents are directed towards molecules involved in the RAS-RAF-MEK-ERK-MAPK pathway, which is frequently over-activated (132). **BRAF inhibitors** have shown the most striking effects among many tested substances. The FDA approved the use of the specific BRAF inhibitor vemurafenib in 2011, after a phase III clinical trial had shown a highly significant increase of survival upon vemurafenib compared to dacarbazine treatment (133). Moreover, patients receiving BRAF inhibitors showed a response rate of 48% and tumour size was reduced drastically. After around 6 month, however, most patients suffer from a relapse of the disease caused by drug resistance of the cancer cells (7,134). Most likely, this resistance occurs due to melanoma cell heterogeneity, selective pressure exerted by BRAF inhibitor treatment and stimulation of alternative pathways of MAPK activation (134). Moreover, BRAF inhibitors paradoxically activate the MAPK pathway in healthy cells. This off-target effect may explain the frequent appearance of keratoacantomas and sqamous cell carcinomas on the skin of treated patients (135). Thus, the combined

therapeutical inhibition of BRAF and downstream MEK are currently under investigation (132). Furthermore, components targeting other frequent mutations in melanomas, such as mutations of KIT, NRAS and PTEN, are in development (132).

» 3.3.3 Systemic Immunotherapy

IFNα has been demonstrated to have a multitude of effects on malignancies, as it inhibits proliferation, induces apoptosis and modulates immune responses. The latter property is characterised by a boost of DC function, enhanced antigen presentation and the promotion of Th1 responses (136). IFNα is used as adjuvant therapy of high-risk melanomas with lymph node involvement or after resection of tumours with increased thickness. Although in these settings high dose IFNα-2b prolonged recurrence-free survival, there is limited data demonstrating an overall survival benefit (126).

IL-2 is an immune regulatory cytokine that has a variety of effects on the immune system, including the activation of T and NK cells. The mechanism behind its anti-tumour activity is, however, not fully understood. High dose IL-2 treatment has the potential of inducing durable responses or complete regression of melanomas (137). Unfortunately, only a small number of patients can profit from high dose IL-2, as response rates evaluated in a set of clinical trials were low (137-140). Moreover, treatment with high dose IL-2 is limited by its severe toxic effects, such as arrhythmias, hypotension and impacts on liver and kidney function, yielding a 1-2% mortality rate (126).

Anti-CTLA-4 treatment has revolutionised melanoma therapy since its approval by the FDA in 2011. Blockage of the inhibitory effect of CTLA-4 on T cells was thought to improve effector T cell responses towards cancer cells (141). In contrast, a more recent study showed that the increased effector T cell activity resulted from anti-CTLA-4-mediated depletion of Tregs in the tumour tissue. (142). Treatment with the anti-CTLA-4 antibody ipilimumab was demonstrated to substantially elevated overall survival rates in phase III clinical trials (143,144). Notably, ipilimumab induced long-term survival in a small fraction of treated patients, some of which had ceased treatment (137). Anti-CTLA-4 treatment causes severe autoimmune adverse effects, including colitis, dyspnea, anemia, hypopituitarism, hypophysitis and rash (143).

Another “immune check-point” is targeted by antibodies binding programmed cell death 1 (PD-1), which leads to enhanced T cell responses. Such antibodies are currently being tested in phase III clinical trials and show promising results (137).

»3.4 Modelling melanoma in mice

Investigation of molecular aspects of development and progression of melanomas, and preclinical testing of therapeutic strategies require animal models. The mouse is one of the most frequently used species for this purpose. The modelling of murine melanomas has been achieved by using genetically modified animals, xeno-transplatation models and syngeneic transplantation models, which are reviewed by Becker and colleagues (145). The discovery of common mutations in melanomas allowed the development of genetically modified animal models targeting driver genes in melanomas. In these mice, melanomas arise spontaneously or can be induced, for example by UV radiation or chemical substances (146). Xeno-transplantation models utilise immune compromised mice as hosts for human melanomas. This approach has been shown to successfully model melanoma properties, such as invasiveness and metastasis formation. Due to their immune suppression they are, however, not suitable to study cancer immunity. Syngeneic transplantation models on the other hand have proven useful to investigate the immune response to melanomas (145). One of the most frequently used cell lines is the B16 melanoma, which is also subject of this study. B16 cells originate from a spontaneous melanoma of C57BL/6 origin and different variants have been selected due to their invasive and metastatic properties (147). One variant, termed B16F10, was described to have a high survival and growth potential *in vivo* and formed more lung metastases when injected intravenously (i.v.), compared to other variants (147). The fact that the B16 line grows in C57BL/6 mice makes it ideal for immunological studies, as there are many genetically modified mice, generated to suit that purpose. Due to the paucity of MHC class I molecules on B16 cells this model was regarded to be poorly immunogenic. The discovery of melanoma-associated antigens and clonally expanded T cells within the tumour, however, demonstrate that the immune system does respond to B16 cells (145). The most commonly used models for the transplantation of B16 cells are the subcutaneous (s.c.) inoculation, which is thought to model a melanoma, and the artificial metastasis lung model, in which B16 cells are injected intravenously (148). B16 tumours have been used to investigate a variety of therapeutic components, some of which have thereafter been successful in clinical trials. One of the most frequently examined therapies is the treatment with IL-12.

»4 Interleukin-12

The pro-inflammatory cytokine IL-12 was first described as an NK cell and T cell activatory factor (149,150), inducing the production of IFNγ (149). It is secreted by APCs and phagocytes, activated by stimulation of PRRs, as observed for Toll-like receptors, which recognise a multitude of microbial patterns. (151). Optimal production is achieved when the APC is primed by additional signals, such as IFNγ (152), IL-15 (153) or CD40-CD40-ligand interaction (154). Conversely, IL-10 and TGF-β have been found to potently inhibit the production of IL-12 (155,156), illustrating the tight regulation of IL-12 secretion. IL-12 consists of a light (p35) and a heavy chain (p40), which by covalent binding form the active 70kD heterodimer (150,157,158). Whereas the p35 subunit was found to be expressed ubiquitously, p40 expression is confined to immune cells (158). The p40 subunit is, however, not exclusive for IL-12 as it together with the molecule p19 is part of the IL-23 molecule (159). The two cytokines also share receptor chains as the IL-12 receptor (IL-12R) consists of IL-12Rβ1 and IL-12Rβ2, while the IL-23 receptor comprises IL-12Rβ1 and IL-23R (160-162). These overlaps may explain why the function of the two cytokines was initially described as “similar as well as distinct” (159). Particularly in anti-tumour immune responses, these two cytokines play opposing roles (163). The IL-12R is mainly detected on pre-activated T and NK cells (160) and has more recently also been identified on subsets of ILCs (50). Similar to secretion of the cytokine, receptor expression is strictly regulated. Whereas low levels of the IL-12R can be detected on the innate, resting NK cells (164), T cells require activation through TCR engagement to express the IL-12R (165). Moreover, IL-12Rβ2 expression was found to correlate with responsiveness to IL-12 and could be induced by IL-12 and IFNγ signalling, while IL-4 was a potent inhibitor (165). Receptor engagement results in the activation of the Janus kinase (Jak) – signal transducer and activator of transcription (STAT) pathway, leading to STAT4 phosphorylation, which mediates the main cellular effects of IL-12 (166).

The effects of IL-12 have predominantly been investigated in lymphocytes, but IL-12 signalling may also affect other cell populations. For instance, IL-12 has been shown to act on APCs, which may imply an autocrine feedback mechanism for the secretion of IL-12 (167). IL-12R engagement on NK and T cells promotes proliferation and enhances cytotoxic effector functions (149,150,168). IL-12-induced cytotoxicity was later shown to be mediated mainly by triggering perforin and granzyme expression and by upregulating adhesion molecules (reviewed in Trinchieri et al., 2003 (164)). IL-12 is also a powerful driver of T helper cell differentiation. Polarisation towards the IFNγ secreting T_H1 phenotype was shown

to be promoted by IL-12 signalling (169), while T_H2 responses were suppressed (170). IL-12 stimulation is, however, not absolutely required for the T_H1 fate and rather potentiates the development by providing proliferative signals and inducing IFNγ. Instead, the transcription factor T-bet was sufficient for the stimulation of IFNγ and generation of Th1 cells in the absence of IL-12 and STAT4 signalling (171). Moreover, T-bet was found to induce the expression of the IL-12Rβ2 chain and thereby increases the responsiveness to IL-12 (172).

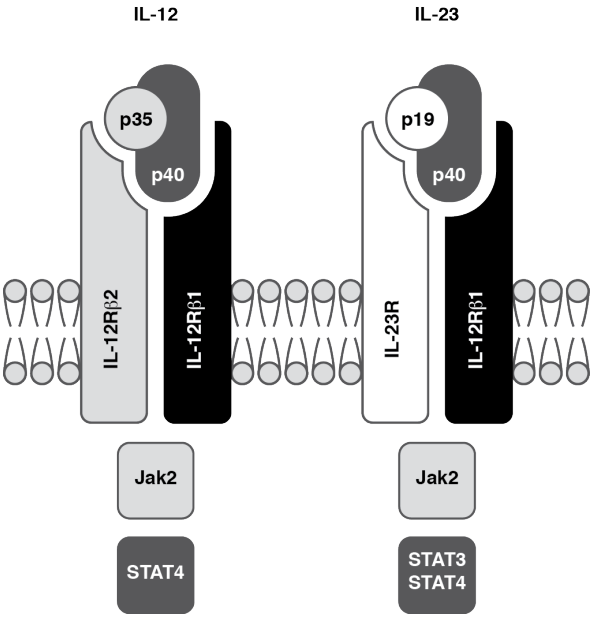


Illustration 4: IL-12 and IL-23, their receptors and signal transmitters. The IL-12 and IL-23 cytokines both contain the p40 heavy chain, which dimerises with p35 to form IL-12 or with p19 to form IL-23. Upon binding to the correspondent receptors, which entail the IL-12Rβ2 subunit, IL-12 mainly signals over STAT4, while IL-23 stimulation predominantly leads to STAT3 and STAT4 phosphorylation. Adaptation from Trinchieri et. al. (265)

IL-12 stimulates lymphocytes to secrete a variety of cytokines, such as TNF, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-2 (164) and is a particularly potent inducer of IFNγ (149,164). IFNγ itself has a plethora of cellular effects, which are reviewed by Boehm et al. and Schroeder et al. (173,174). These effects are initiated upon signalling through the IFNγ receptor (IFNγR), primarily leading to STAT1 phosphorylation. Apart from responses similar to IL-12 as promotion of cytotoxicity and T_H1 polarisation, IFNγ activates myeloid cells and their anti-microbial functions, such as the release of nitric oxide (NO) and ROS. In addition, IFNγ induces antigen presentation by controlling various steps of the MHC class I and class II pathways and thereby promotes the activation of $CD4^+$ and $CD8^+$ T cells. By stimulating the IL-12 secretion by APCs (152) IFNγ solidifies the generation of T_H1 responses.

For IFNγ the cellular responses are, however, not restricted to the leukocyte compartment as for IL-12, but the IFNγR is expressed on all somatic cells. IFNγ signalling, for instance, upregulates adhesion molecules such as ICAM-1 (175) and VCAM-1 (176) and enhances

secretion of chemokines, leading to facilitated leukocyte trafficking. Notably, IFNγ also inhibits proliferation and induces apoptosis, which is why it can limit immune responses, inhibit angiogenesis and suppress growth of tumours (173,174).

»4.1 IL-12 in tumour immunity

There is evidence that IL-12 has a role in the immune surveillance of tumours and interferes with cancer development. Initially, mice lacking the p40 subunit were utilised to test the effect of IL-12 on early tumourigenesis. In a model of MCA-induced fibrosarcoma, for instance, tumours growth was increased in p40-deficient animals compared to controls (177). The discovery of IL-23, sharing both p40 subunit and IL-12Rβ1 chain, however, put these results into perspective. Using mice deficient for the p35 subunit, Langowski and colleagues observed that the lack of IL-12 increased susceptibility to chemically induced papillomas. In contrast, p19 knock out mice developed fewer papillomas, demonstrating the tumour promoting effect of IL-23 (163). In another study, utilising mice deficient for p35, UVB exposure led to increased numbers of skin tumours and transition from benign lesions to carcinomas was enhanced (178). In addition, IL-12Rβ2-deficient mice (*IL12rb2*$^{-/-}$) have been shown to develop plasmacytomas and lung carcinomas, which arose completely spontaneous (179). While these data suggest the importance of endogenous IL-12 in suppressing tumour establishment, many more studies focused on the therapeutic potential of exogenously administered IL-12.

The first evidence of treatment efficiency was contributed in 1993, when Brunda and colleagues demonstrated the anti-tumour effect of systemically delivered IL-12 in a series of transplantable tumour models (180). The effect appeared to be dose-dependent and induced suppression of tumour growth even when treatment was initiated 14 days after tumour cell inoculation (180). Further studies supported these results and not only transplantable tumours but also carcinogen (181) and genetically induced malignancies (182) were demonstrated to be sensitive to IL-12 treatment (183). Nastala and colleagues were the first to establish the important role of IL-12 stimulated IFNγ for subsequent rejection of tumours (184). Many more studies confirmed confirming this observation, by both depleting IFNγ signalling genetically and by using blocking antibodies (reviewed by Colombo and Trinchieri, 2002 (183)).

The various tissue specific responses to IFNγ have briefly been introduced above. In the context of tumour immunity, however, the anti-proliferative effect, particularly that on tumour vessels, deserves some more attention. The anti-angiogenic effect of IL-12 was first

described by Voest et al. and was attributed to increased IFNγ production (185). Later, Kanegane and colleagues associated the IFNγ inducible chemokines CXCL-10 (IP-10) and CXCL-9 (Mig) with the IL-12-mediated inhibition of angiogenesis and vascular damage, causing tumour necrosis. By neutralising IFNγ or both CXCL-10 and CXCL-9, the tumour suppression by IL-12 was abolished. They concluded that inhibition of vessel formation was essential for the subsequent anti-tumour response (186). Furthermore, IFNγ-induced inhibition of VEGF (187), VEGF receptor 3 (188) and MMP-9 (187,189) production were correlated with the inhibition of both angiogenesis and tumour progression, upon IL-12 administration . Both direct and indirect effects of IFNγ on endothelial cells were suggested to inhibit their growth (188). Several studies implied that a lymphocyte-endothelial cell interaction is needed to elicit the full anti-angiogenic effect (189,190). The upregulation of ICAM-1 and VCAM-1 is thought to further promote this response, by mobilising immune cells to the tumour (189). In contrast, another report demonstrated that the tumour cells themselves secreted CXCL-10 upon IFNγ signalling, generating the anti-angiogenic activity (191).

IFNγ-independent rejection of tumours upon IL-12 treatment has also been reported. In the C26 colon carcinoma model, for instance, the anti-tumour effect of IL-12 partially relied on GM-CSF-secreting $CD4^+$ T cells (192). Moreover, IL-12 induced the IFNγ-independent release of perforin by NK cells or T cells, suppressing lung B16 melanomas (75) and orthotopic GL-261 gliomas (193).

T cell responses were considered critical for the IL-12-induced anti-tumour immunity in a number of reports (180,184,193). Whereas the anti-tumour response towards s.c. renal adenocarcinomas (RENCA) was reduced upon depletion of $CD8^+$ T cells (180), tumour suppression required both $CD8^+$ and $CD4^+$ T cells in subcutaneous MCA-207 sarcomas (184) and intracranial Gl261 gliomas (193). Other studies, rather focused on metastasis models, however, suggested the involvement of NKT cells (194) or observed a RAG2-independent mechanism, in which NK cells were sufficient for IL-12-induced tumour suppression (195). Smyth and colleagues reconciled this controversy by showing that the contribution of NKT and NK cells to tumour rejection varied, dependent on the tumour model and IL-12 treatment regimen. For i.v. injected B16 lung tumours NK cells, eliminated by anti NK1.1 depletion, were required for the anti-tumour immunity upon high doses of IL-12. In contrast, the effect of low doses or delayed treatment start was reduced in Jα281 knock out mice, lacking NKT cells (177). Park et. al. later demonstrated that IL-12 suppressed growth of s.c. tumours and liver metastases in CD1-deficient mice, independently of NKT cells. Whereas liver metastases were controlled by NK cells, s.c. tumours were still rejected in anti-

NK1.1 treated RAG2-deficient mice (*Rag2*$^{-/-}$), suggesting that the site of tumour implantation determined the mechanism of IL-12-mediated suppression. Moreover, in *Rag2*$^{-/-}$ mice deficient for the common gamma chain of the IL-2 receptor (*Rag2*$^{-/-}$*Il2rgc*$^{-/-}$; lacking all lymphocyte populations) the IL-12-induced anti-tumour response was abolished. Thus, the authors concluded that a distinct, non-T non-B non-NK, but gamma chain-dependent cell was required for the rejection of s.c. B16 melanomas (196). In a transfer model of engineered tumour-antigen specific CD8$^+$ T cells secreting IL-12, Kerkar and colleagues found that CD11b$^+$ cells of the myeloid compartment responded IL-12 administration (197). This partly IFNγ-dependent stimulation of myeloid cells led to increased antigen cross-presentation, which allowed the transferred T cells to accumulate in the tumour tissue and thereby potentiated the effect of treatment (197).

» 4.1.1 Innate lymphoid cells in IL-12-induced tumour rejection

Our group previously investigated the mechanism of IL-12-induced tumour suppression in a model of s.c. transplanted B16F10 cells, which were modified to secrete IL-12Fc (B16F10-IL-12Fc). The fusion of IL-12 with the crystallisable fragment of mouse immunoglogulin G3 (Fc) was performed to increase the half-life time, the local containment and, hence, the effect of IL-12. Similar to the study of Park et al. (196), IL-12Fc-induced suppression of B16F10 tumours was independent of adaptive immune cells and NK cells (198). Instead, a subtype of ILCs, dependent on the transcription factor RORγt, was observed to mediate tumour rejection. To identify RORγt-dependent ILCs a transgenic system was utilised, in which RORγt expressing cells were irreversibly labelled with the fluorophore enhanced yellow fluorescent protein (eYFP). For this, mice expressing the Cre recombinase under the promoter of the RORγt encoding gene *Rorc* (*Rorc*-Cre) (199) were used. *Rorc*-Cre animals were further bred to *Rosa26*-stop$^{fl/fl}$-eYFP reporter mice, which express eYFP under the ubiquitously active *Rosa26* promoter upon Cre-mediated excision of a loxP flanked stop cassette. In *Rorc*-Cre x *Rosa26*-stop$^{fl/fl}$-eYFP fate map mice (here referred to as *Rorc*$^{fm+}$), RORγt expression results in continuous expression of eYFP (RORγt^{fm+} cells), which is how RORγt-dependent lineages can be traced.

The co-transfer of purified lineage negative RORγt^{fm+} ILCs together with B16F10-IL-12 cells into an IL-12Rβ2-deficient host (*Il12rb2*$^{-/-}$) elicited a potent suppression of tumour growth. As B16F10 cells were shown not to bear the IL-12R on their surface, in this system, the transferred populations were the only IL-12 responsive cells. Thus, the transfer of RORγt^{fm+} ILC demonstrated that these cells were sufficient to induce a potent anti-tumour response upon IL-12 activation. In contrast, growth of B16F10-IL-12Fc tumours was not impaired upon transfer of NK cells (198) or T cells (K. Nussbaum and S. Burkhard, observation). The failure

of splenocytes harvested from RORc-deficient mice at inducing tumour rejection, suggested that RORγt-dependent ILC were not only sufficient, but also necessary for the IL-12-induced anti-tumour immunity (198). Although RORγt^{fm+} ILC secreted high amounts of IFNγ upon IL-12 activation, the tumour suppression was found to be IFNγ independent. B16F10-IL-12Fc tumours showed increased levels of the adhesion molecules ICAM-1 and VCAM-1 on the tumour vasculature and the accumulation of immune cells was observed. This suggested that the promotion of leukocyte migration into the tumour tissue could be involved in the mechanism behind IL-12-induced tumour rejection (198).

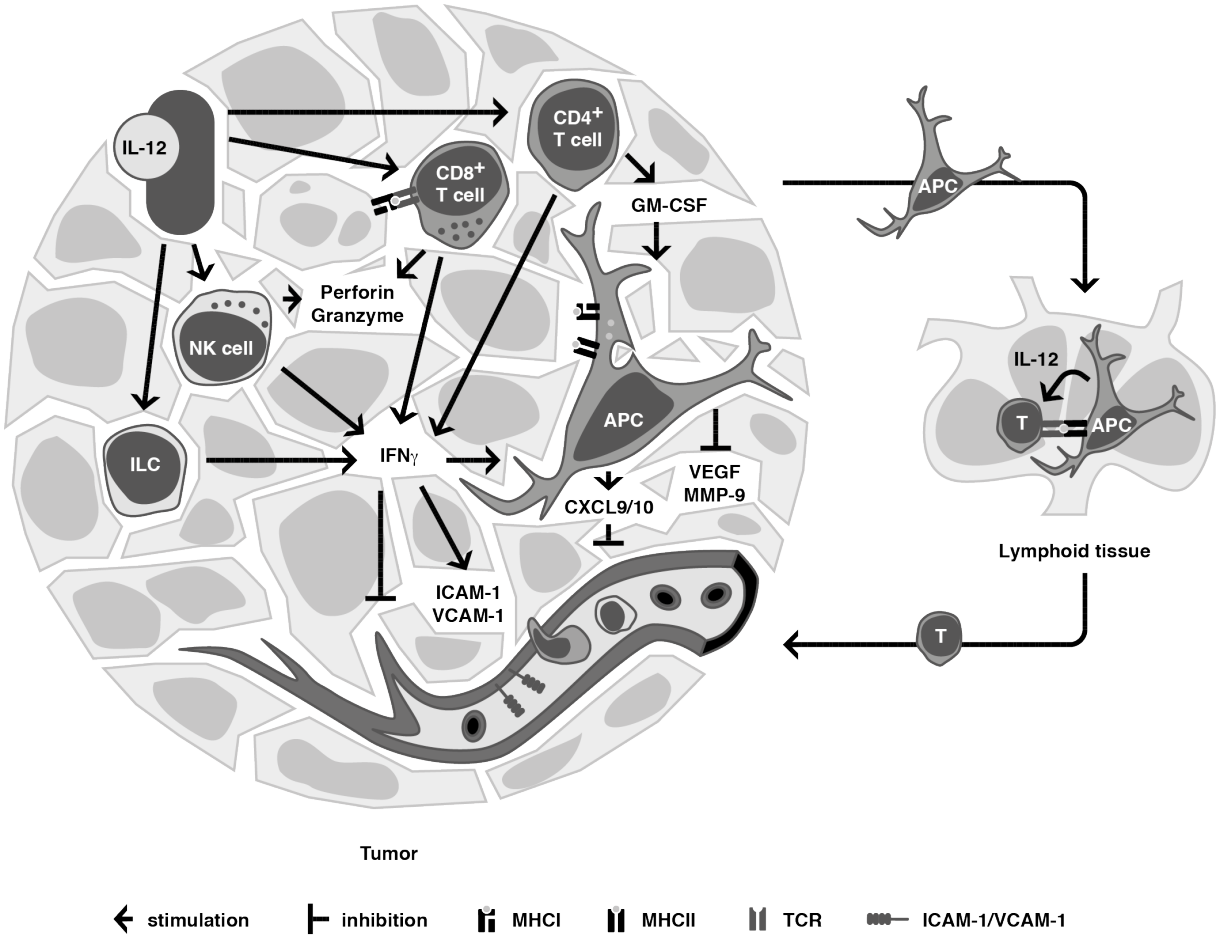


Illustration 5: The effects of IL-12 stimulation in the tumour tissue. IL-12 primarily acts on the lymphoid compartment, involving NK cells, T cells and ILCs and enhances their IFNγ secretion. The majority of the tumour-suppressing pathways, occurring upon IL-12 treatment, are mediated by IFNγ. In turn, IL-12 and IFNγ stimulate cytotoxic effector functions by NK cells and CD8$^+$ T cells, which involve the production of perforin and granzyme. IFNγ further directly impacts the tumour vasculature by upregulating ICAM-1 and VCAM-1 and suppressing angiogenesis. The enhanced adhesion molecule expression is thought to promote leukocyte recruitment to the tumour site. IFNγ activates myeloid cells, inducing CXCL9 and CXCL10 secretion and suppressing VEGF and MMP-9 production, processes which promote the anti-angiogenic effect. IL-12 treatment leads to enhanced antigen presentation and cross-presentation by APCs, further promoting the cytotoxic activity of CD8$^+$ T cells and cytokine release by CD4$^+$ T cells. T helper cell-derived GM-CSF has been observed to be involved in tumour suppression upon IL-12 stimulation. T: T cell. Illustration adapted from Tugues et al., 2014 (206).

»4.1.2 Cancer immunotherapy with IL-12

The success of IL-12 as a cancer therapeutic in preclinical models was promising in matters of future immunotherapy of human cancer patients. In a phase II study conducted by Leonard and colleagues, systemic i.v. IL-12 treatment, however, triggered unexpectedly severe adverse effects (200,201). These side effects included dyspnea, stomatitis, leukopenia, thrombocytopenia, and elevated blood levels of transaminases and bilirubin, and required hospitalisation of some probands. Two patients developed gastrointestinal bleeding with consecutive hypotension or a sepsis-like syndrome with multiorgan failure, in both cases leading to death of the proband (201). Both the toxic effects and the impact on cancer disease were correlated with high and sustained levels of IFNγ (201,202). In general, clinical response rates to i.v. IL-12 therapy appeared moderate, with few probands suffering from renal cancer (201-203), melanoma (203), ovarian cancer (204) and breast cancer (205) benefitting from partial or transient responses. The awareness of these clinical limitations increased the focus on combining IL-12 with other anti-cancer treatments and localised strategies of administration in preclinical and clinical studies. To more specifically target the tumour, recombinant IL-12 has been injected intratumourally and coupled to tumour antigen specific antibodies (immunocytokines). In addition, the delivery of IL-12 encoding DNA by electroporation, hydrodynamic injection, viral vectors, polymeric microspheres or nanoparticles, or transfer of engineered cells, have been utilised to achieve local secretion of IL-12 (reviewed in Tugues et al., 2014 (206)). The advances made in this field indeed led to the success of some clinical studies. Out of 10 patients with cutaneous T cell lymphomas, receiving IL-12 injections s.c. and intratumourally (i.t.), 9 responded and 2 showed complete remission (207). In combination with rituximab, an antibody directed against CD20 expressing B cells, s.c. IL-12 administration yielded a 69% response rate and 26% complete remissions (208).

»5 Innate lymphoid cells

The recently identified family of ILCs is currently attracting much attention. By now, ILCs are recognised to be a heterogeneous group of cells involved in retaining homeostasis and tissue repair. When inadequately activated they can, however, contribute to inflammatory processes. ILCs are localised in lymphoid and non-lymphoid tissues, but predominantly reside at mucosal barriers of the gut, lung and skin. While sharing their morphology with other cells of the lymphoid lineage, they lack rearranged antigen specific receptors and are thought to respond to pathogens promptly. ILCs develop from common lymphoid progenitors and can secrete a variety of T_H cell-related cytokines. Moreover, they mirror T_H cell-associated transcriptional features and surface marker expression, and have therefore been suggested to be evolutionary precursors of adaptive immune cells (reviewed in (50,209,210)).

The discovery of ILC subsets started with the identification of $CD4^+CD3^-$ cells, accumulating in the anlage of murine lymph nodes (211,212). Subsequently, two groups demonstrated that the transfer of such $CD4^+CD3^-$ cells to alymphoplastic mice resulted in the formation of peyers patches (secondary lymphoid structures in the small intestines) (213) and nasal-associated lymphoid tissue (214). Due to their non-redundant role for the formation of secondary lymphoid tissues, they were later termed lymphoid tissue inducer cells (LTis) (215). Subsequently, LTis were identified in human fetal mesenteric lymph nodes but, in contrast to mouse LTis, these cells did not express CD4 (216). Discovery of the essential role for the transcription factor RORγt for LTi generation (217) allowed fate map studies, which revealed that LTis persisted in the gut of adult mice (199). LTis were later also detected in adult spleens, where they were suggested to establish B and T cell segregation (218) and restore the splenic architecture upon viral disruption (219). In addition, adult LTis retained the ability to induce the *de novo* generation of lymphoid tissues (220). In 2009, adult splenic LTis were identified to be an innate source of the cytokines IL-17 and IL-22 (221). At the same time, innate cells expressing RORγt and natural cytotoxicity receptors (NCRs; in this case NKp46 in mouse and NKp44 in human) were detected in the murine gut and human tonsils, and were demonstrated to secrete IL-22 (222-225). These cells were clearly distinct from NK cells, as they showed no or low expression of the NK cell marker NK1.1, and failed to secrete IFNγ and exert cytotoxic effector functions (222,224). Despite some similarities with LTis, these cells did not express IL-17 (222-224). Nevertheless, these discoveries initiated a discussion about a possible lineage relationship between the novel NCR^+ IL-22 producing cells, NK cells and LTis (226,227).

The described populations were ultimately unified in the family of ILCs, due to their innate nature, lymphoid morphology and common requirement for the transcriptional regulator Id2 (inhibitor of DNA binding 2) (209). Id2 prevents the binding of enhancer (E) proteins to E-box sites and thereby hampers the differentiation of precursor cells towards the T and B cell lineage (228). Id2-deficient animals have been demonstrated to lack various subtypes of ILCs, including mature NK cells (229-232). This suggests that a common $Id2^+$ ILC precursor exists, such cells, however, yet have to be identified (233). Two independent groups recently isolated Id2 expressing precursor cells from the murine bone marrow, which could develop into various ILC subsets, but failed to generate conventional NK cells (234,235) or $CD4^+$ LTis (235). Moreover, ILCs require cytokine signalling through the IL-2 receptor subunit gamma. Whereas IL-15 is important for both NK cell development and maintenance (236), the generation of other ILC subsets has been shown to rely on IL-7 signalling (231). Regarding their transcriptional profile and their pattern of cytokine expression, ILCs have been classified into three different groups in both mice and humans, reminiscent of the diverse T_H cell subsets (50). The following paragraphs will summarise characteristics of these groups, focusing on murine ILCs.

» 5.1 Group 1 ILCs

These cells are defined by their capacity to produce IFNγ and comprise NK cells and the recently identified ILC1s. Both populations express the transcription factor T-bet during development. ILC1s also share certain surface molecules with NK cells, such as NKp46 and in some mouse strains NK.1.1 (234). In contrast to NK cells, ILC1s in the gut were shown to lack expression of the integrin CD49b (234). While Klose and colleagues found that ILC1s were reduced in IL-15-deficient mice (234), this was not the case in the study by Fuchs et al. (237). ILC1s characterised in these two studies also diverged regarding expression of eomesodermin (Eomes) (234,237), a transcription factor which in NK cells cooperates with T-bet (238).

Classical group 1 ILCs develop independently of RORγt transcription (234). Phenotype and function of ILC1s can, however, be adopted by RORγt-dependent group 3 ILCs (239-241). This transition involves down-regulation of RORγt, which is why these cells are commonly referred to as “ex-RORγt” ILC3s (239,240). Conversely, they upregulate T-bet, authorising the cells to express IFNγ and NKp46 (240). In the gut, this transition depends on the host microbiota, as germ free mice had lower numbers of $NKp46^+$ ILC3s (240). Interestingly, differentiation towards the ILC1 phenotype is supported by IL-12 and IL-18 signalling, which

further stimulate IFNγ secretion (240,241). Moreover, IL-15 was shown to be critical for the maintenance of NKp46^{+}ILC3 (234). Cells with ILC1 function have been ascribed a protective role in Salmonella enterica and Toxoplasma gondii infections of the gut (234,240).

It is still disputed whether NK cells should be incorporated in the group 1 of ILCs. A study by Boos et al. reported that normal numbers of NK cell progenitors were present in the bone marrow of Id2-deficient mice. The lack of nearly all the mature NK cells indicated that rather than defining NK cell lineage, Id2 was required for maturation (230). Furthermore, NK cells are enabled to exert cytotoxic effector functions, whereas both ILC1s and ILC3s lack this capacity (222,224,234).

»5.2 Group 2 ILCs

The second group of ILCs are characterised by the secretion of the T_H2-associated cytokines IL-5, IL-13 in response to IL-25 and IL-33. Similar to T_H2 cells, ILC2s depend on the transcription factor GATA3 for their development and maintenance (242,243). A recent study by Yagi and collaborators, however, demonstrated that also the development of other ILC subsets required GATA3 expression. Using conditional knock out mice for GATA3, they revealed that both ILC2 and group 3 ILC precursors were decreased in the bone marrow. Whereas the ILC2 population also required GATA3 for its maintenance, group 3 ILCs did not (244). Moreover, transcription factor RORα expression was shown to control ILC2 development (245,246). Functionally, ILC2s are related to immunity against helminth infection, as IL-13 for instance supports worm expulsion, by promoting hyperplasia of mucus producing goblet cells, (232,247). ILC2s can, however, also mediate pathology as shown in models of airway hyper reactivity (248,249).

»5.3 Group 3 ILCs

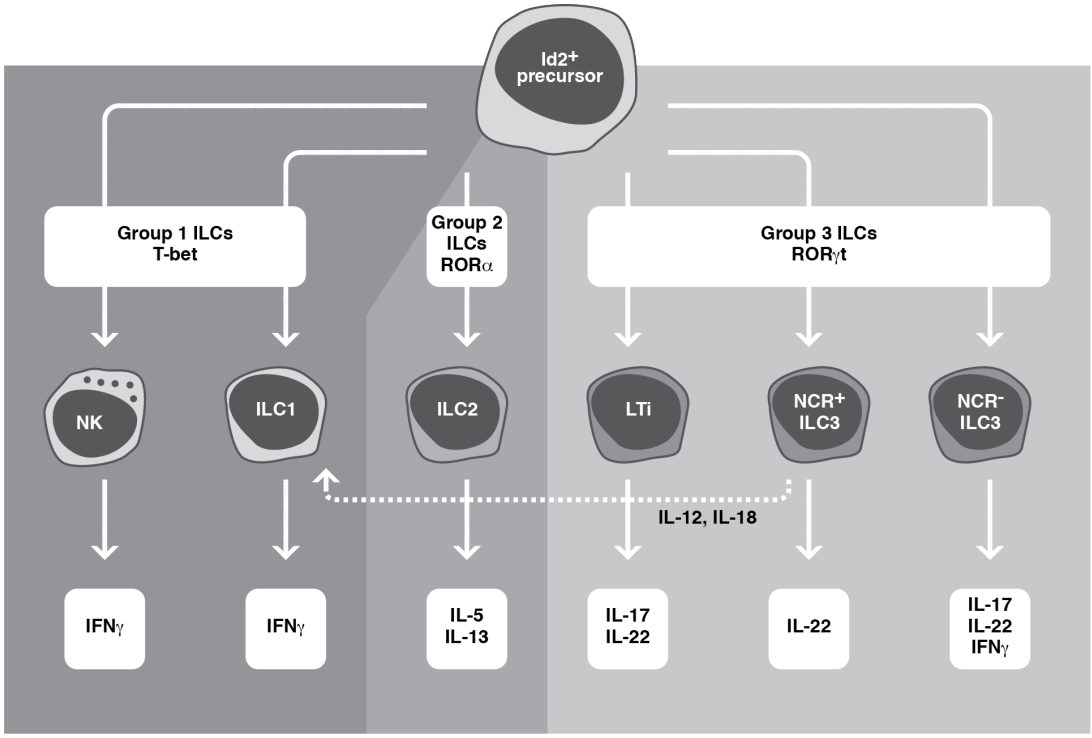
Group 3 ILCs are related by their capacity to secrete the cytokines IL-22 and IL-17, and their responsiveness to IL-23. They rely on RORγt expression for their development and function. This group is composed of LTis and ILC3s, the latter is further subdivided into NCR^{+} and NCR^{-} ILC3s.

LTis organise the development of secondary lymphoid tissues, a process which is achieved by a complex interplay between LTis and stromal organiser cells (250). This crosstalk critically relies on LTis expressing lymphotoxin α1β2, which binds to the lymphotoxin β receptor on the stromal cells. Signalling induces the expression of chemokines (CCL19,

CCL21 and CXCL13) and adhesion molecules, such as ICAM-1, VCAM-1 and mucosal vascular addressin cell adhesion molecule 1 (MADCAM-1). Thereby lymphocytes are not only attracted to the site of lymphoid tissue development, but are also retained there (250). While lymph nodes and peyers patches are formed before birth, other mucosal-associated lymphoid tissues (MALT) develop postnatally, some of which arise in the absence of LTis (250). The interaction of the immune system with the microbiota has been suggested to influence the development of certain postnatal lymphoid structures (251).

The term **ILC3s** incorporates group 3 ILCs that are functionally distinct from LTi cells. Some of these cells have been described to express the NCR NKp46 and are the main source of IL-22 in the gut after birth (252). Moreover, a subtype of $CD4^+$ ILCs were demonstrated to contribute to intestinal IL-22 (253). IL-22 in turn has been shown to maintain epithelial barrier integrity upon exposure to pathogens (254,255). Thus, the lack of NCR^+ILC3s results in a lower expression of anti-microbial molecules (224) and increased susceptibility to intestinal infection (222). ILC3s thereby seem to control the microbiota, but there is also evidence that the commensal bacteria influence IL-22 producing ILC3s. How exactly pathogens achieve this and how they may affect development, maintenance and function of ILC3s is not yet completely understood (256). Just like the transition of ILC3s towards ILC1 function the generation of NCR^+ILC3s was demonstrated to rely on T-bet expression. In mice deficient for T-bet the population of NCR^+ILC3s is decreased, leading to compromised IL-22 production (257). ILC3s have also been found to promote inflammation in a colitis model (258). This population was characterised by the secretion of IL-17 and IFNγ and did not express NKp46 (NCR^-ILC3s) (258). In comparison to NCR^+ILC3s these cells did not depend on T-bet and potently induced innate colitis in mice deficient for both T-bet and RAG2 (259). Furthermore, the accumulation of human NCR^- ILC3s and increased IL-17 secretion was detected in the inflamed intestine of patients suffering from Crohns disease (260).

A great challenge for the research on recently identified ILC populations remains the fact that no exclusive ILC markers have been identified so far. Thus, ILCs have mainly been defined by their lack of lineage markers (261). Furthermore, they have been characterised by the expression of surface molecules related to hematopoietic progenitors (SCA-1 and cKit), T cells (CD4, Thy-1) and NK cells (NKp46, NK1.1) (50). In table 1 the distribution of phenotypical markers over distinct ILC populations is summarised.



	NK	ILC1	ILC2	LTi	NCR+ ILC3	NCR- ILC3
IL-12Rβ2	+	+	-	-	-	-
IL-23R	-	-	-	+	+	+
Thy-1	+/-	ND	+	+	+	+
IL-7Rα	+/-	+	+	+	+	+
c-Kit	-	-	+	+	+	-
NKp46	+	+/-	-	-	+	-
CD4	-	-	-	+	+/-	-

Illustration 6: ILC development into three groups and their expression patterns. Illustration summarising the development of different ILC subsets from a common Id2$^+$ precursor cell their requirement for distinct transcription factors and their individual cytokine profiles. The dashed arrow indicates the transition of NCR$^+$ILC3s towards ILC1 phenotype and function. The table below depicts the expression profile of the various ILC subsets as characterised up to now. ND: not determined, +: expressed, -: not expressed; +/-: may or may not be expressed. Illustration is adapted from Spits et al., 2013 (50).

»5.4 Innate lymphoid cells and cancer

Few studies have dealt with the role of ILCs in cancer. A report by Shields et al. described that tumour-derived CCL21 promoted the accumulation of LTis and the development of lymphoid structures in the tumour margins. These structures were absent in RORγt-deficient mice, supporting the necessity for LTis. The formation of such lymphoid tissue was correlated with accelerated tumour growth and development of a tolerogenic environment. For instance, Tregs were abundant in the tumour and TGF-β levels increased, while suppressed levels of IFNγ and fewer tumour-antigen specific CD8$^+$Tcells were detected (262). Another study by Kirchberger and collaborators supported the hypothesis that group 3

ILCs acted as tumour promoters. In a model of bacteria and carcinogen-induced colorectal carcinoma, IL-22 secretion by NCR$^-$ILC3s was shown to maintain tumour growth. After the appearance of epithelial dysplasia, neutralisation of IL-22 and anti-Thy-1 antibody-mediated depletion of ILC3s reduced tumour burden in experimental mice. Moreover, IL-22 signalling was shown to increase the proliferation of dysplastic epithelial cells (263). In addition, in a mammary carcinoma model the accumulation of IL-13 secreting ILC2s has been linked to the enhanced tumour growth upon IL-33 administration (264). Thus, the study by our group deviates from other reports by ascribing an anti-tumour activity to RORγt-dependent ILCs (198). Although the upregulation of ICAM-1 and VCAM-1 in the tumour tissue was reminiscent of LTi function, tumour suppression was independent of lymphotoxin β receptor signalling (198).

V Specific aims of the thesis

Immune modulation by IL-12 treatment remains an interesting approach to combat cancer. Many questions regarding the precise mechanism, by which IL-12 achieves the elimination of tumours have, however, been left unanswered. Particularly, the implication of various immune cell types responding to IL-12 in this context is conflicting. Our group previously identified the tumour suppressive potential of RORγt-dependent ILCs. The role of such ILCs was, however, only tested in an artificial model of IL-12 secreting tumour cells, providing the B16F10 tumours with IL-12 during tumour tissue establishment. Thus, it is of interest, whether the action of RORγt-dependent ILCs is still required for the rejection upon therapeutical IL-12 intervention after tumour formation. Moreover, the recent awareness of various subtypes within the ILC3 compartment asks for the further characterisation of phenotype and function of the tumour suppressive ILCs. This description may facilitate the understanding of what molecular and cellular players are involved in the ILC-mediated anti-tumour response elicited by IL-12.

In this thesis I investigated the time dependency of IL-12 treatment by comparing the mechanisms of tumour rejection upon early preventive with late therapeutic treatment of s.c B16F10 tumours. The involvement of different cell types in tumour rejection upon late therapeutic administration of IL-12 was examined, using transgenic mice.

RORγt-dependent ILC populations within spleen and small intestines were characterised regarding phenotype and tumour suppressive function. In the appendix we elaborate on how to improve the gating strategy on ILCs.

VI Results

»6 IFNγ is required for the rejection of late, but not early IL-12-treated tumours.

The mechanism by which IL-12 rejects tumours has been suggested to depend on the timing of IL-12 treatment (177). For the continuous administration, as in B16F10-IL-12Fc tumours, growth suppression was shown to rely on the activation of RORγt-dependent ILCs through IL-12Fc (198). In such tumours, IL-12Fc was present from early tumour formation onwards. We aimed to assess whether RORγt-dependent ILCs were also required upon late therapeutic delivery of IL-12. Therefore, we treated B16F10 tumours from early or late time points during tumour growth. 2 x 10^5 B16F10 cells were inoculated subcutaneously, leading to the establishment of a solid tumour, which reached withdrawal criteria towards day 21. In one group of mice, administration of IL-12Fc was started simultaneously with B16F10 inoculation (day 0), mimicking the "treatment" modality of IL-12Fc secreting B16F10 tumours. The second group received IL-12Fc after day 7, when a small tumour had formed, in order to replicate a therapeutic intervention (Illustrated in Figure 1 A). In both groups, IL-12Fc or PBS was administered three times a week via s.c. injections before, and i.t. after establishment of a palpable lesion. As the IL-12-mediated anti-tumour effect was shown to be mainly induced by stimulating IFNγ secretion in previous studies (183), we compared tumour suppression in wild type (WT) animals and mice deficient for the IFNγ receptor (*Ifngr1*$^{-/-}$).

In WT animals, early and late IL-12Fc treatment led to a substantial reduction of tumour growth (Figure 1 B). On day 7 upon B16F10 inoculation, early IL-12Fc treated tumours were significantly smaller than tumours from controls, in WT and *Ifngr1*$^{-/-}$ mice. Until day 21, these tumours had merely reached 10 to 15% of the size of PBS administered tumours. In late treated WT mice the tumour size was reduced compared to controls. This treatment modality failed in *Ifngr1*$^{-/-}$ mice, where similar tumour sizes were measured upon late IL-12Fc or PBS administration. To adjust for the overall accelerated tumour growth in *Ifngr1*$^{-/-}$ in contrast to WT mice, day 21 tumour sizes of the treated groups were compared to the corresponding PBS treated tumours to evaluate the effect of treatment. While late IL-12Fc administration yielded a 70 % reduction of tumour size in WT mice, tumours in *Ifngr1*$^{-/-}$ mice were only 6% smaller than control tumours. (Figure 1 B).

Thus, IFNγ signalling is critical for the anti-tumour immunity upon late IL-12Fc injection while early treated tumours were rejected in its absence. We concluded that the time point of

treatment initiation influences the quality of the subsequent anti-tumour response. Thus, we hypothesised that distinct cellular and molecular responses may be involved in the rejection of early compared to late treated tumours.

Figure 1

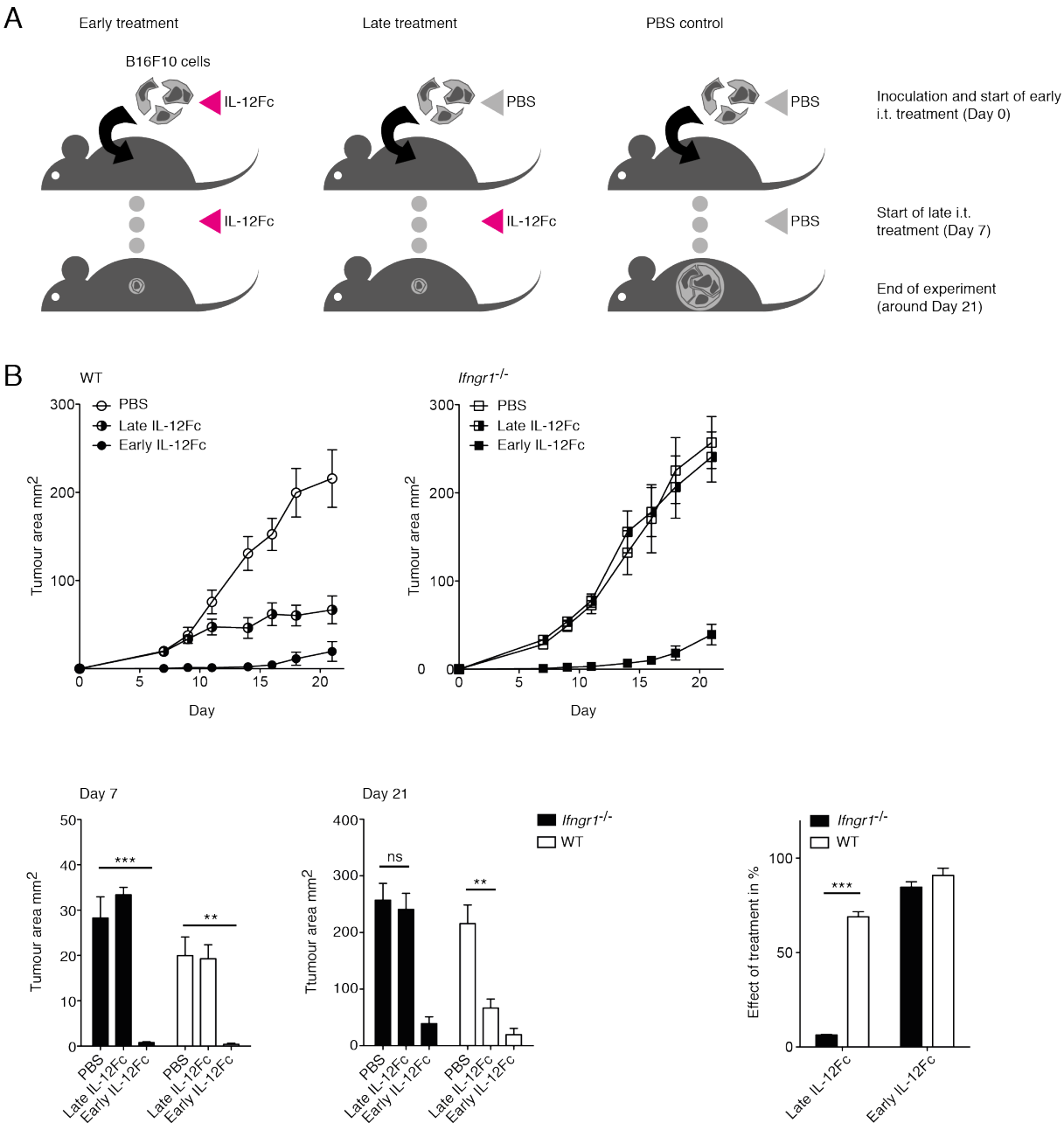


Figure 1. The anti-tumour response upon late IL-12Fc treatment requires IFNγ signalling. (A) Illustration of early and late treatment with IL-12Fc or administration of PBS to controls. (B) Top row: Tumour growth curve upon early and late IL-12Fc treatment in WT and *Ifngr1*$^{-/-}$ mice. Bottom row: Left: Quantification of tumour size in WT and *Ifngr1*$^{-/-}$ animals on day 7 and day 21 after inoculation of tumours corresponding to top row. Right: Effect of IL-12Fc treatment measured by the ratio of IL-12Fc treated versus control tumour size for WT and *Ifngr1*$^{-/-}$ mice corresponding to top row. (Data is pooled from 3 independent experiments n=15 (n refers to the number of individual mice used in the experiment), mean ± S.E.M)

Figure 2

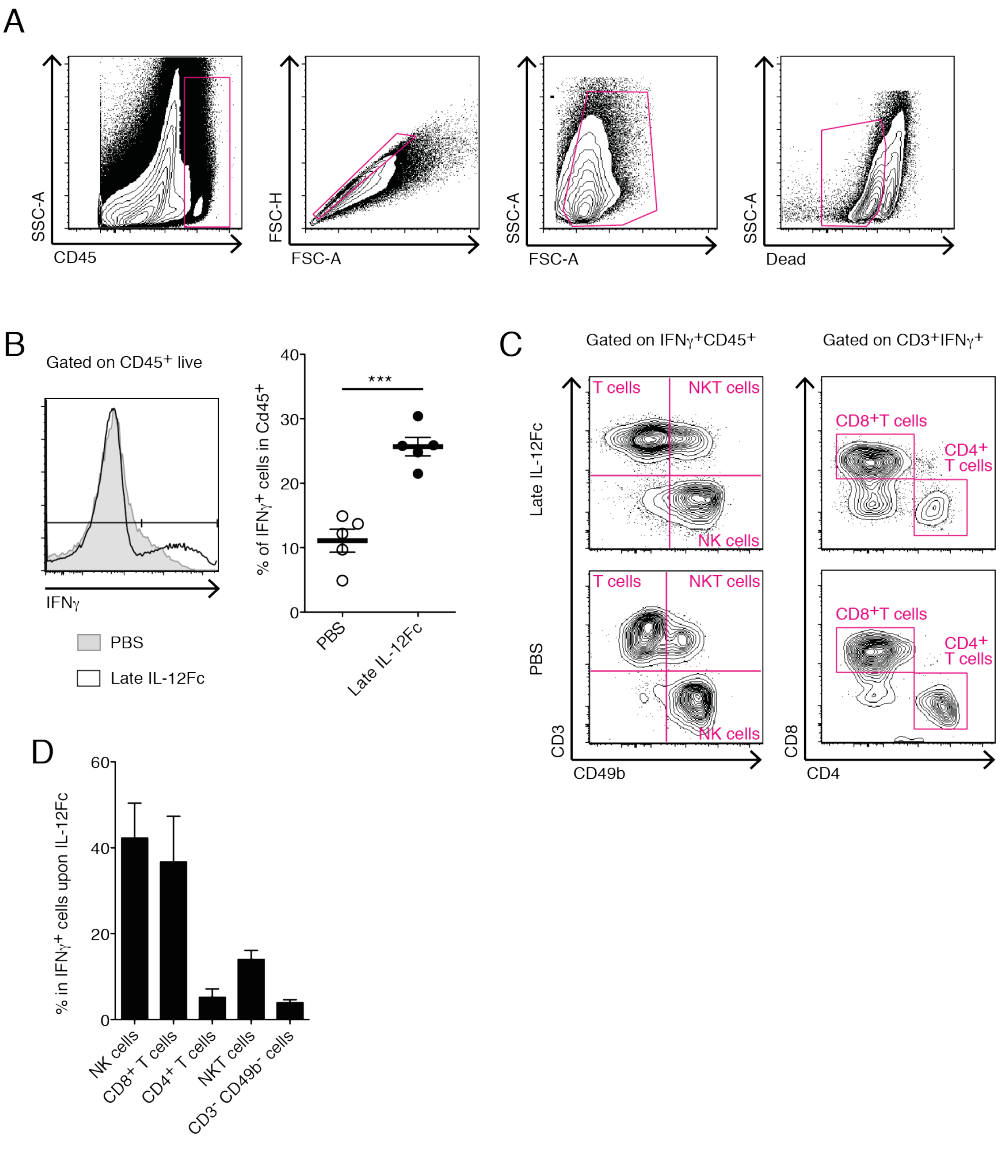


Figure 2. NK cells and CD8$^+$ T cells are the main source of IFNγ in late IL-12Fc treated tumours. (A) Representative FACS plots showing gating on CD45$^+$, singlets, leukocytes and exclusion of dead events in tumours on day 10 of growth. Similar gating strategies were used for all analyses of leukocytes in the tumour tissue. (B) Representative histogram and quantification of IFNγ$^+$ cell frequency within CD45$^+$ live events isolated from late IL-12Fc treated compared to PBS administered tumours. (C) Representative graphs showing gating on CD3$^+$ versus CD49b$^+$ - and within the CD3$^+$ events CD4 versus CD8 - in IFNγ$^+$CD45$^+$ events of PBS administered or late IL-12Fc treated tumours. (D) Quantification of the frequencies of cells within the IFNγ$^+$CD45$^+$ population shown in C. (n=5, mean ± S.E.M, experiment performed once)

»7 The mechanism upon late IL-12Fc treatment

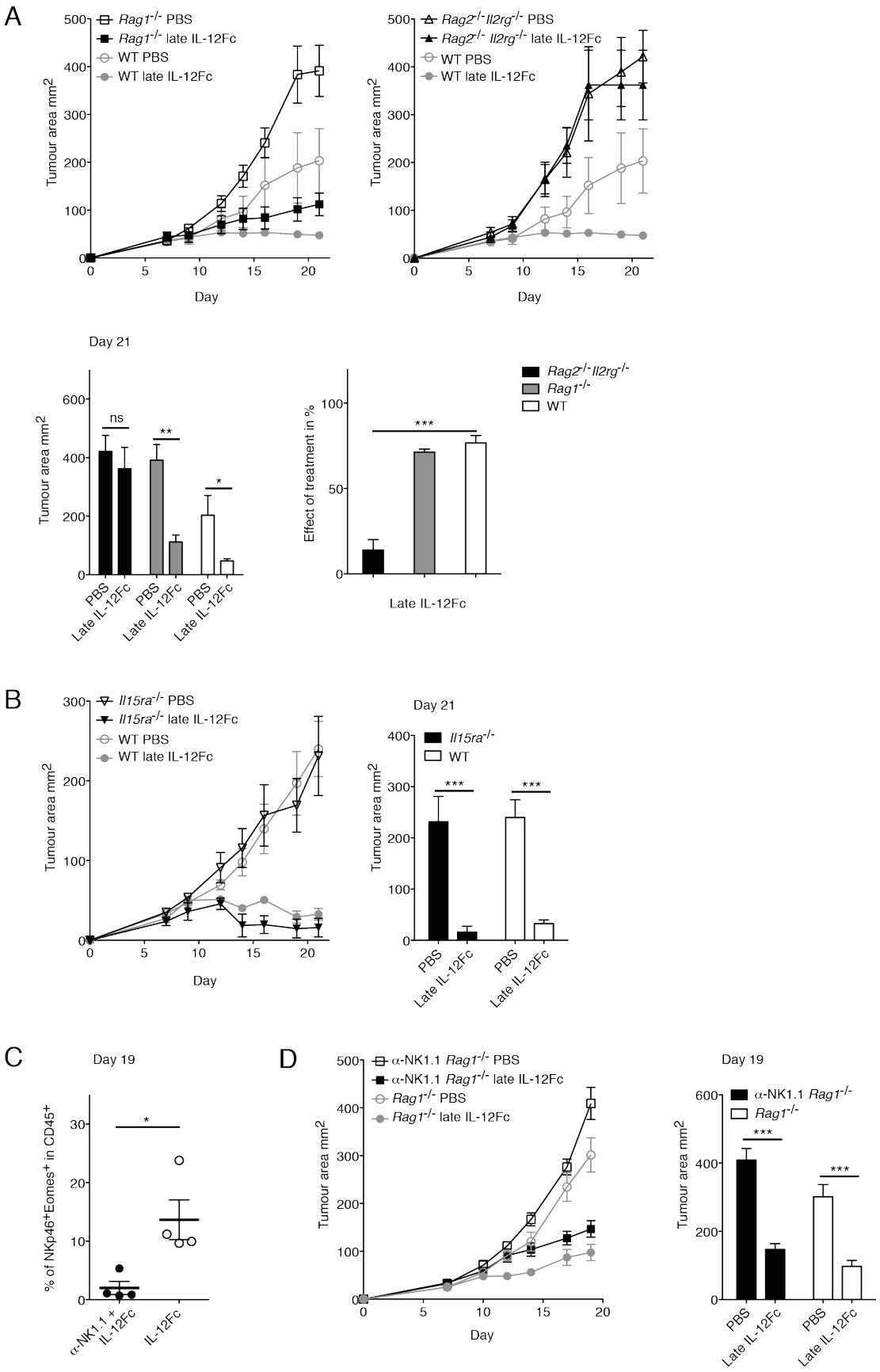
»7.1 NK cells and CD8$^+$ T cells are the main cellular source of IFNγ

Due to the necessity of IFNγ for tumour control upon late IL-12Fc treatment, we aimed to identify the cellular source of IL-12Fc stimulated IFNγ. We examined leukocytes within tumours on day 10 upon B16F10 inoculation, when IL-12Fc would generally have started to affect the tumour growth. This allowed us to explore the first phase of B16F10 rejection. Leukocytes were isolated from IL-12Fc treated and control tumours and analysed for their IFNγ secretion by flow cytometry. The gating on intratumoural leukocytes involved exclusion of doublets, cellular debris and dead cells from the CD45 expressing leukocytes (Figure 2 A).

Within this population IL-12Fc treatment doubled the frequency of IFNγ-secreting cells, compared to control tumours (Figure 2 B). Upon IL-12Fc stimulation, IFNγ was solely produced by intratumoural lymphocytes as no myeloid cells – characterised by their high expression of CD11b – were detected within the IFNγ$^+$CD45$^+$ leukocytes (Data not shown). The IFNγ$^+$ population was then further divided by gating on CD4$^+$ and CD8$^+$ CD3$^+$CD49b$^-$ T cells, CD3$^+$CD49b$^+$ NKT cells and CD3$^-$CD49b$^+$ NK cells (Figure 2 C). CD8$^+$ T cells and NK cells were identified as the main source of IFNγ in the tumour tissue as they together accounted for 80% of the IFNγ$^+$ fraction (Figure 2 D). While IL-12Fc treatment boosted the IFNγ production, the contribution of distinct cell subsets to the IFNγ secretion was not altered in comparison to control tumours (Data not shown).

Figure 3. IL-12Fc-induced tumour suppression upon late treatment does not require T cells or NK cells but depends on the common gamma chain signalling. (A) Growth of PBS administered or late IL-12Fc treated B16 F10 tumours in *Rag1*$^{-/-}$ mice and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice compared to WT. Tumour sizes were assessed on day 21 and the effect of treatment (ratio of IL-12Fc treated versus PBS administered tumour) was elaborated (n=5-6, experiment performed twice) (B) Tumour growth curve and corresponding tumour sizes on day 21 of late IL-12Fc treated or PBS administered *Il15ra*$^{-/-}$ mice compared to control (n=5, experiment performed twice). (C) Quantification of NKp46$^+$Eomes$^+$ NK cell frequencies within tumours of anti-NK1.1 IL-12Fc treated or PBS administered mice upon late IL-12Fc administration. (D) Tumour growth upon anti-NK1.1 treatment of *Rag1*$^{-/-}$ mice and corresponding tumour area measured on day 19 (n=4, experiment performed once). (mean ± S.E.M)

Figure 3


A
Rag1-/- PBS
Rag1-/- late IL-12Fc
WT PBS
WT late IL-12Fc
Tumour area mm2
Day
Rag2-/-Il2rg-/- PBS
Rag2-/- Il2rg-/- late IL-12Fc
Day 21
ns
**
*
PBS
Late IL-12Fc
Effect of treatment in %
***
Late IL-12Fc
B
Il15ra-/- PBS
Il15ra-/- late IL-12Fc
C
Day 19
% of NKp46+Eomes+ in CD45+
α-NK1.1 + IL-12Fc
IL-12Fc
D
α-NK1.1 Rag1-/- PBS
α-NK1.1 Rag1-/- late IL-12Fc
Rag1-/- PBS
Rag1-/- late IL-12Fc

»7.2 NK and T cells are dispensable for suppression of late treated tumours.

Since T cells and NK cells were the main contributors to IFNγ secretion, we next assessed, whether these cells was required for successful repression of tumour growth by IL-12Fc upon late treatment. To study the role of T cells, tumour growth was monitored in RAG1-deficient mice (*Rag1*$^{-/-}$), lacking T and B cells, and compared to tumour growth in WT and *Rag2*$^{-/-}$*Il2r*$^{-/-}$ mice. In *Rag2*$^{-/-}$*Il2r*$^{-/-}$ mice, the tumour suppressing effect of IL-12Fc was abolished (Figure 3 A). The accelerated tumour growth in both PBS treated *Rag1*$^{-/-}$ and *Rag2*$^{-/-}$*Il2r*$^{-/-}$ mice compared to WT, suggested that tumour growth was controlled by T cells. In the presence of IL-12Fc, however, *Rag1*$^{-/-}$ mice were still able to mount an anti-tumour immunity comparable to WT mice (Figure 3 A). We therefore conclude that tumour rejection relies on lymphoid cells, but that T cells are dispensable in this context.

In order to investigate the role of NK cells in tumour control, mice that lack IL-15 receptor expression (*Il15ra*$^{-/-}$), deficient in mature NK cells, were challenged with B16F10 tumours. Those mice efficiently suppressed tumour growth upon injection of IL-12Fc to the same extent as WT mice (Figure 3 B). Thus, we hypothesised that T and NK cells may compensate for the absence of the other to secrete sufficient amounts of IFNγ for successful suppression of tumour growth. In a preliminary experiment, we attempted to eliminate NK cells in *Rag1*$^{-/-}$ mice, by anti-NK1.1 antibody mediated depletion. The weekly monitoring of depletion efficiency in the blood of experimental animals showed a drastic reduction of NK cells (Data not shown). The lack of peripheral NK cells was further supported by a low frequency of NKp46^{+}Eomes^{+} cells in the tumours of anti-NK1.1 treated mice (Figure 3 C). Even in the absence of both NK and T cells, tumour growth was potently suppressed upon late IL-12Fc treatment (Figure 3 D).

»7.3 Tumour growth upon late IL-12Fc treatment is not controlled by RORγt-dependent ILCs

The intact anti-tumour response in mice lacking NK and T cells, suggested a role for residual innate lymphoid cells upon late IL-12Fc treatment. The potent anti-tumour activity of RORγt-dependent ILCs may, thus, also impact tumour growth in the therapeutical setting. The observation that such ILCs secreted IFNγ upon IL-12 stimulation *in vitro* (198), further supported this hypothesis. To study RORγt-dependent ILCs, we utilised a RORc-eGFP reporter mouse (*Rorc*$^{rep+}$), in which all RORc transcribing cells simultaneously express enhanced green fluorescent protein (eGFP). Mice homozygous for this allele (referred to as *Rorc*$^{-/-}$) fail to express functional RORγt, as the coding sequence for eGFP is inserted into

exon 1 of the RORc locus, (215). Thus, in *Rorc*$^{-/-}$ mice the IL-12Fc-mediated anti-tumour response could be studied in absence of RORγt-dependent lineages. *Rorc*$^{-/-}$ and *Rag1*$^{-/-}$ animals were crossed to obtain *Rorc*$^{-/-}$*Rag1*$^{-/-}$ mice, which differed from *Rag1*$^{-/-}$ mice only by the absence of RORγt-dependent ILCs.

Figure 4

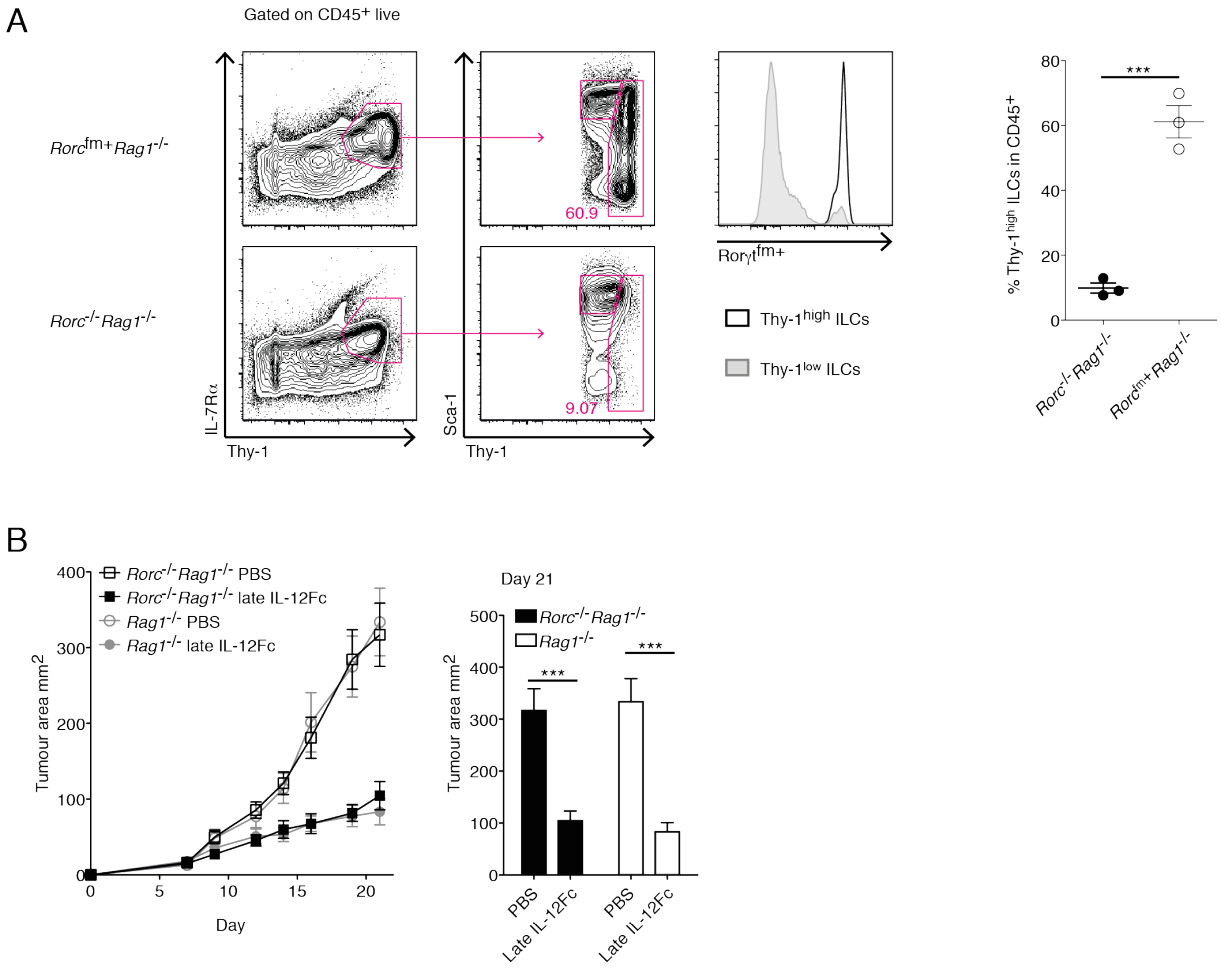


Figure 4. IL-12Fc suppresses tumour growth independent of RORγt expressing ILCs. (A) Gating strategy on Thy-1^{+}IL-7Ra^{+} ILCs in the gut of naïve *Rorc*$^{-/-}$*Rag1*$^{-/-}$ and *Rorc*$^{fm+}$*Rag1*$^{-/-}$ mice pre-gated on CD45^{+} live events. (General gating strategy for gut lymphocytes is shown in figure 12.) RORγt^{fm} expression on Thy-1^{low} Sca-1^{+} and Thy-1^{high} cells within the Thy-1^{+}IL-7Ra^{+} population in *Rorc*$^{fm+}$*Rag1*$^{-/-}$ mice. Frequencies of these subpopulations in *Rorc*$^{-/-}$*Rag1*$^{-/-}$ and *Rorc*$^{fm+}$*Rag1*$^{-/-}$ mice (n=3, experiment performed twice). (B) Growth curve and tumour sizes on day 21 post inoculation of late IL-12Fc treated or PBS administered tumours in *Rorc*$^{-/-}$*Rag1*$^{-/-}$ mice and *Rag1*$^{-/-}$ controls. (n=5, experiment performed twice) (mean ± S.E.M)

First we confirmed the lack of RORγt-dependent ILCs in *Rorc*$^{-/-}$*Rag1*$^{-/-}$ mice and thereby also assessed their requirement for the transcription factor RORγt. For this reason, ILCs from *Rorc*$^{-/-}$*Rag1*$^{-/-}$ mice were compared to cells from *Rorc*$^{fm+}$ fate map animals bred to *Rag1*$^{-/-}$ mice (*Rorc*$^{fm+}$*Rag1*$^{-/-}$), in which only RORγt-dependent ILCs were RORγt^{fm+}. For the flow

cytometric analysis, the lamina propria of the small intestine was selected, due to the abundance of RORγt-dependent ILCs in this location. Total ILCs were defined as Thy-1^{+}IL-7Rα^{+} cells within the CD45^{+} live compartment (Figure 4 A). ILCs were then further subdivided into a Thy-1^{low}Sca-1^{+} and a Thy-1^{high} population. In *Rorc*$^{fm+}$*Rag1*$^{-/-}$ mice the Thy-1^{low}Sca-1^{+} population contained very few RORγt^{fm+} cells, whereas, the majority of Thy-1^{high} cells expressed YFP (Figure 4 A). We therefore concluded that the Thy-1^{high} cells referred to the RORγt-dependent subgroup among ILCs. As expected, this RORγt-dependent Thy-1^{high} ILC population was absent from the small intestine of *Rorc*$^{-/-}$*Rag1*$^{-/-}$ mice, which is illustrated by a drastic decrease in frequency compared to control (Figure 4 A).

Rorc$^{-/-}$*Rag1*$^{-/-}$ mice were challenged with B16F10 tumours, which were IL-12Fc treated from day 7 onwards. The lack of RORγt-dependent ILCs did not affect the anti-tumour response upon IL-12Fc treatment as illustrated by comparable tumour sizes in *Rag1*$^{-/-}$ and *Rorc*$^{-/-}$*Rag1*$^{-/-}$ mice on day 21 of tumour growth (Figure 4 B).

Thus we concluded that RORγt-dependent ILCs were dispensable for the tumour rejection upon late IL-12Fc treatment. Thereby, the cellular responses underlying the tumour suppression upon early or late IL-12Fc treatment seemed to diverge.

» 8 Cellular targets of IL-12-induced IFNγ in late treated tumours

» 8.1 The effects of IFNγ on tumour myeloid cells

Thus far, our data suggested that IL-12Fc-stimulated lymphocytes secrete IFNγ, which was required for the subsequent anti-tumour immunity. Interestingly, the elimination of various lymphocyte populations did not impair tumour suppression. Apart from suggesting that the depleted cells were dispensable for a solid IFNγ induction, these experiments show that neither NK cells nor cells of adaptive immunity were required to respond to IFNγ for the IL-12Fc-induced anti-tumour response to occur. Since IFNγ promotes myeloid cell functions, such as antigen presentation and secretion of ROS and NO (174), we hypothesised that this compartment may also be involved in the rejection of IL-12Fc treated tumours.

» 8.1.1 IL-12-induced IFNγ activates of monocyte-derived cells in tumours

In order to characterise the intratumoural myeloid compartment we first aimed to determine at which time points leukocytes responded to IL-12Fc-induced IFNγ. Thus, tumours from WT and *Ifngr1*$^{-/-}$ mice were analysed for infiltrating leukocytes early (day 10) and late (day 19) during tumour rejection upon late IL-12Fc treatment. Whereas in WT animals, IL-12Fc

administration provoked a 4-fold increase of intratumoural $CD45^+$ leukocyte numbers, this was not observed *Ifngr1*$^{-/-}$ mice on day 10 of tumour growth (Figure 5 A). In comparison, day 19 tumours showed less infiltrates and the number of $CD45^+$ cells was comparable in IL-12Fc or PBS administered WT and *Ifngr1*$^{-/-}$ mice (Figure 5 A). Thus, the further flow cytometric analysis of myeloid cells was performed on day 10 of tumour growth, when IL-12Fc initiated the IFNγ-dependent accumulation of leukocytes.

Granulocytes were identified by their high expression of Ly6G within the $CD45^+$ compartment (Figure 5 C). The majority of $CD11b^+$ $Ly6G^-$ cells were observed to express high levels of Ly6C, a characteristic marker for monocytic cells. A large proportion of the $Ly6C^{high}Ly6G^-$ fraction stained positive for MHC class II ($MHCII^+$), indicating that intratumoural monocytes differentiated to monocyte-derived macrophages or DCs. Of the $MHCII^+$ population some cells expressed iNOS, suggesting elevated NO secretion. Moreover, we identified APCs by their expression of CD11c and MHC class II within $CD45^+$ cells in the tumour. The majority of these APCs also expressed Ly6C and CD11b, indicating that they were the progeny of monocytes. In contrast, a small proportion of $CD11b^-$ DCs was detected (Figure 5 C). Further, we quantified the frequencies and total numbers of tumour myeloid populations in IL-12Fc treated or PBS administered WT and *Ifngr1*$^{-/-}$ mice. The frequency of $Ly6C^{high}Ly6G^-$ cells was significantly elevated in IL-12Fc treated WT mice, whereas these changes were not observed in the absence of IFNγ signalling (Figure 5 D). In contrast the frequency of $CD11b^-$ DCs was decreased in IL-12Fc treated WT mice, compared to *Ifngr1*$^{-/-}$ mice. Although the proportion of $Ly6G^+$ granulocytes seemed to decline upon IL-12Fc administration to WT animals, this observation was not significant. The total number of monocytes, and monocyte-derived macrophages and DCs within IL-12Fc administered tumours was substantially elevated in an IFNγ-dependent manner. The accumulation of $MHCII^+$ and $iNOS^+$ cells upon treatment in WT but not in *Ifngr1*$^{-/-}$ mice suggested a general activation of myeloid cell function by IFNγ. In contrast, the numbers of non-monocytic cells, such as granulocytes and $CD11b^-$ DCs were not influenced by IL-12Fc treatment (Figure 5 D). The increase of monocytes and monocyte-derived cells and decrease of other myeloid populations was further confirmed in tumours on day 19 of growth (Data not shown). Due to the reduced leukocyte numbers in such tumours these changes were, however, generally less pronounced compared to day 10 tumours.

In conclusion, the accumulation of monocytes and their progeny in the tumour tissue correlated with the IL-12-induced anti-tumour effect.

Figure 5

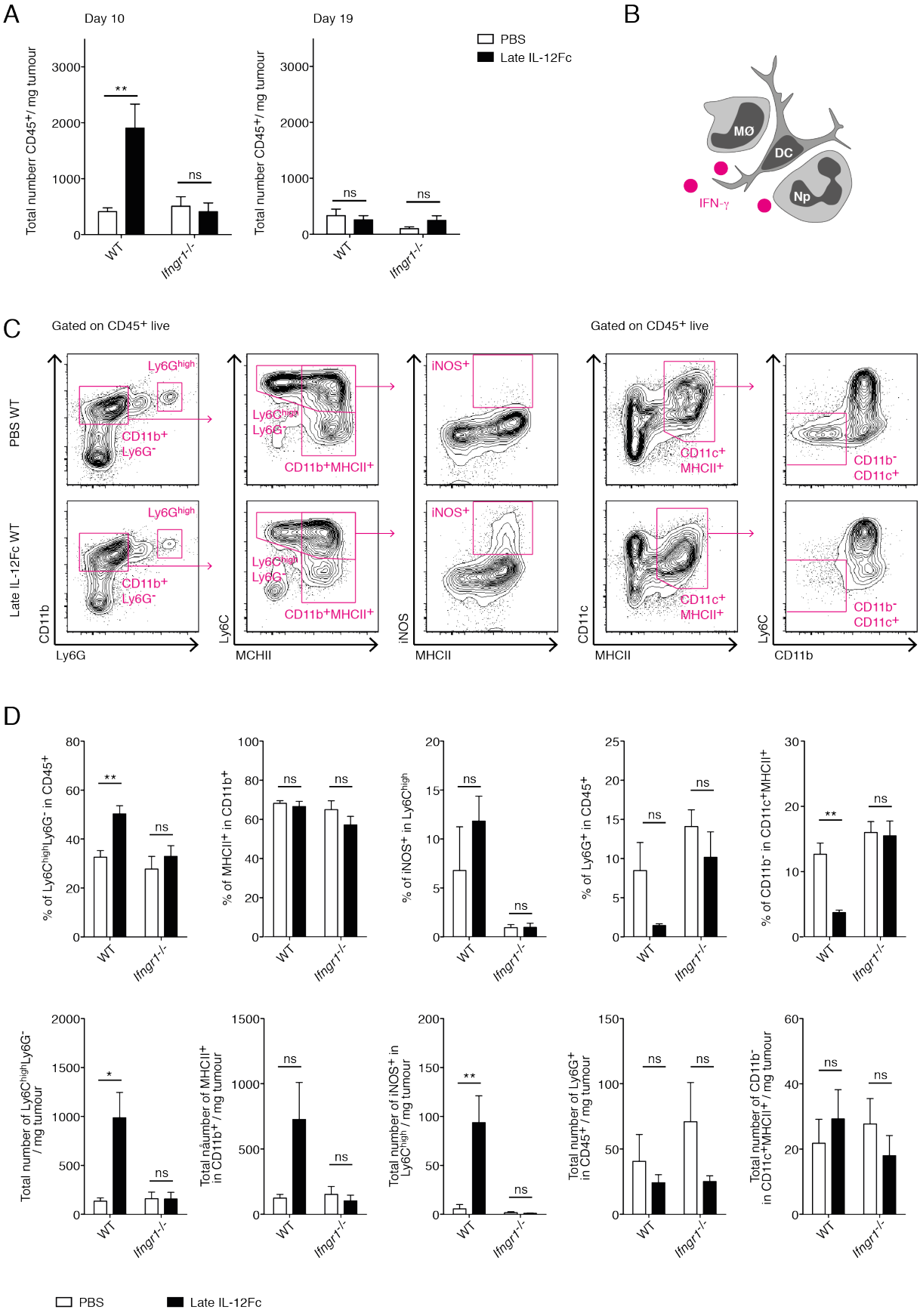

A
Day 10
Day 19
PBS
Late IL-12Fc
Total numberr CD45+/ mg tumour
Total number CD45+/ mg tumour
WT
Ifngr1-/-
ns
B
MØ
DC
Np
IFN-γ
C
Gated on CD45+ live
Gated on CD45+ live
PBS WT
Late IL-12Fc WT
Ly6Ghigh
CD11b+ Ly6G-
Ly6Chigh Ly6G-
CD11b+MHCII+
iNOS+
CD11c+ MHCII+
CD11b- CD11c+
CD11b
Ly6G
Ly6C
MCHII
iNOS
MHCII
CD11c
MHCII
Ly6C
CD11b
D
% of Ly6Chigh Ly6G- in CD45+
% of MHCII+ in CD11b+
% of iNOS+ in Ly6Chigh
% of Ly6G+ in CD45+
% of CD11b- in CD11c+MHCII+
Total number of Ly6Chigh Ly6G- / mg tumour
Total number of MHCII+ in CD11b+ / mg tumour
Total number of iNOS+ in Ly6Chigh / mg tumour
Total number of Ly6G+ in CD45+ / mg tumour
Total number of CD11b- in CD11c+MHCII+ / mg tumour
PBS
Late IL-12Fc

Figure 5. IL-12Fc treatment leads to IFNγ-dependent recruitment of monocytic cells to the tumour. (A) Bar graph displays total numbers of intratumoural CD45$^+$ leukocytes on day 10 and 19 of tumour growth in WT and *Ifngr1*$^{-/-}$ mice (n=4-5, mean ± S.E.M, experiment performed twice (day 10) and three times (day 19)). (B) Illustration suggesting the activating role of IFNγ on the myeloid cell compartment. (C) Gating strategy on different myeloid cell populations in PBS administered or late IL-12Fc treated WT tumours on day 10 of growth. (D) Quantification of frequencies (top row) and total numbers (bottom row) of myeloid cells in IL-12Fc treated or PBS administered WT and to *Ifngr1*$^{-/-}$ tumours on day 10 of growth. (n=4-5, mean ± S.E.M, experiment performed once)

»8.1.2 CCR2 driven migration of myeloid cells is not required for the anti-tumour effects of late IL-12Fc treatment

To determine whether monocytes and monocyte-derived cells were essential for the anti-tumour response upon late IL-12Fc treatment, we made use of mice deficient for the chemokine receptor CCR2 (*Ccr2*$^{-/-}$) (266). This receptor binds the monocyte chemoattractant protein-1 (MCP-1/CCL2) and mediates the chemotaxis of monocytes and lymphocytes (267). CCR2 is implied in monocyte trafficking during homeostasis (267,268) and is particularly essential for the recruitment of monocyte-derived cells to inflammatory tissues (35) and tumours (101).

In *Ccr2*$^{-/-}$ mice, the IL-12Fc-initiated anti-tumour effect was comparable to suppression of B16F10 tumours in WT controls (Figure 6 A). To investigate the impact of CCR2-deficiency on the infiltrating myeloid cells we isolated leukocytes from the tumours on day 22 of growth. *Ccr2*$^{-/-}$ tumours showed a drastic decrease in frequency and total number of Ly6C^{high}Ly6G$^-$ monocytic cells (Figure 6 B and C). The reduction of monocytes was, however, accompanied by an increase in the frequency of Ly6G$^+$ granulocytic cells, which was more pronounced upon IL-12Fc treatment (Figure 6 B and C). This counterbalance may explain the comparable total numbers of CD45$^+$ infiltrating leukocytes in WT and *Ccr2*$^{-/-}$ mice (Figure 6 D). The lack of Ly6C^{high}Ly6G$^-$ monocytes influenced the presence of monocyte-derived cells illustrated by the reduction in frequency and absolute numbers of CD11c$^+$ cells in the tumour (Figure 6 C and D). In contrast, immunofluorescent analysis revealed that F4/80$^+$ cells were frequently detected in tumours form *Ccr2*$^{-/-}$ mice on day 13 of growth. (Figure 6 E). Thus, we assume that CCR2-independent F4/80$^+$ tumour macrophages are present within B16F10 tumours.

We concluded that the IL-12Fc-induced anti-tumour response is independent of CCR2, but may still involve Ly6G$^+$ granulocytes or residual myeloid cells observed in *Ccr2*$^{-/-}$ tumours. Tissue resident macrophages within the subcutis may, for instance, populate the tumour independently of CCL2 signalling or accumulate by local proliferation.

Figure 6

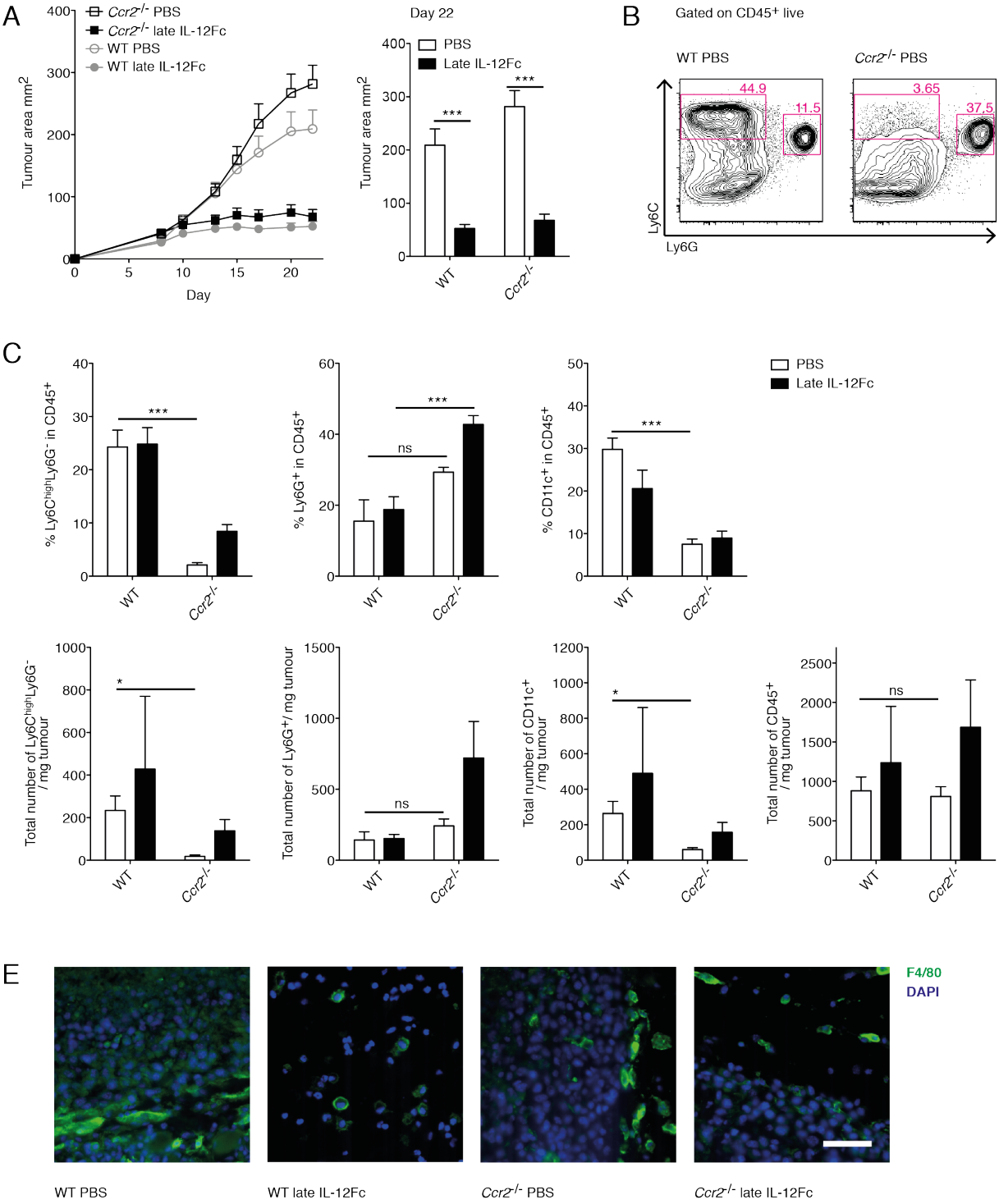


Figure 6. CCR2-dependent migration of monocytes is dispensable for the anti-tumour effect upon late IL-12Fc treatment. (A) Growth of IL-12Fc treated or PBS administered tumours in *Ccr2*$^{-/-}$ and WT mice and quantification of tumour area on day 22 (pooled from 3 independent experiments, n=13-15, mean ± S.E.M) (B) Gating on Ly6C^{+}Ly6G^{-} and Ly6G^{-} cells within CD45^{+} live cells in PBS administered *Ccr2*$^{-/-}$ and WT tumours. (C) Top row: Frequencies of Ly6C^{+}Ly6G^{-}, Ly6G^{-} and CD11c^{+} cells within the CD45^{+} population of IL-12Fc treated or PBS administered tumours from *Ccr2*$^{-/-}$ and WT mice. Bottom row: Total numbers of the populations depicted in top row. (n=3-7, mean ± S.E.M, experiment performed twice) (D) Immunofluorescent staining for F4/80 combined with nuclear stain 4',6-diamidino-2-phenylindole dilactate (DAPI, blue) of late IL12Fc treated or PBS administered tumours from *Ccr2*$^{-/-}$ and WT mice on day 13 of growth (scale bar refers to 50 μm, n=5, experiment performed twice).

»8.1.3 The direct response of LysM expressing cells to IFNγ is not required for the late IL-12Fc-dependent anti-tumour immunity

We further aimed to examine the role of mature macrophages and granulocytes during the IL-12Fc-mediated anti-tumour response. These cells are characterised by high expression of Lysozyme 2 (LysM), an enzyme with bacteriolytic activity (269,270). Moreover, LysM expression was demonstrated to gradually increase during differentiation and upon activation of myeloid cells (271). LysM expressing cells were targeted by utilising mice that express the Cre recombinase under the LysM promoter (*LysM*-Cre^{+}) (272). *LysM*-Cre animals were bred to mice expressing conditional alleles of the IFNγ receptor (*Ifngr*$^{fl/fl}$) to obtain *LysM*-Cre^{+}*Ifngr*$^{fl/fl}$ mice. In these mice the Cre recombinase deletes the loxP site flanked exons 4-6 of the IFNγR gene (273), whereby LysM expressing myeloid cells become unresponsive to IFNγ.

Figure 7

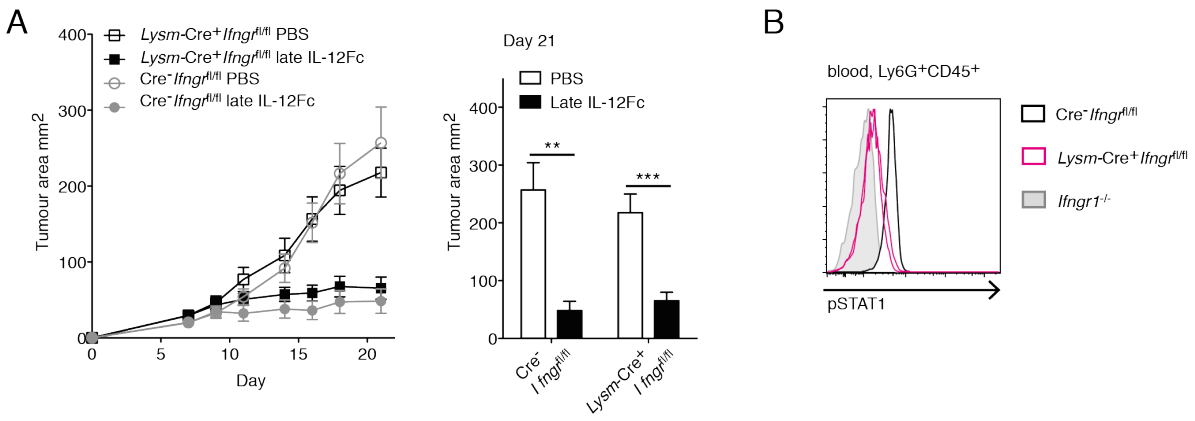


Figure 7. The lack of IFNγ signalling on LysM expressing cells does not impact the anti-tumour response upon late IL-12Fc treatment. (A) Left: Growth of IL-12Fc treated or PBS administered tumours in *LysM*-Cre^{+}*Ifngr*$^{fl/fl}$ mice and Cre^{-}*Ifngr*$^{fl/fl}$ littermates. Right: quantification of tumour area on day 21-post inoculation. (Data pooled from two experiments n=10-11, mean ± S.E.M) (B) Histogram showing pSTAT1 staining on Ly6G+CD45+ cells in the blood of naïve Cre^{-}*Ifngr*$^{fl/fl}$, *LysM*-Cre^{+}*Ifngr*$^{fl/fl}$, and *Ifngr1*$^{-/-}$ mice (n=2; experiment performed once).

We then compared the tumour growth upon late IL-12Fc treatment in *LysM*-Cre^{+}*Ifngr*$^{fl/fl}$ mice to their Cre^{-}*Ifngr*$^{fl/fl}$ littermates. *LysM*-Cre^{+}*Ifngr*$^{fl/fl}$ mice did not show an impairment of the IL-12Fc-induced anti-tumour immunity and tumour sizes were comparable to Cre^{-}*Ifngr*$^{fl/fl}$ controls (Figure 7 A). A preliminary experiment indicated that Ly6G^{+} granulocytes in the blood were less responsive to IFNγ, as STAT1 phosphorylation (pSTAT1) was decreased in *LysM*-Cre^{+}*Ifngr*$^{fl/fl}$ compared to Cre^{-}*Ifngr*$^{fl/fl}$ mice (Figure 7 B). pSTAT1 staining intensities in granulocytes from *LysM*-Cre^{+}*Ifngr*$^{fl/fl}$ and *Ifngr1*$^{-/-}$ mice nearly overlapped, suggesting successful recombination upon Cre expression in those cells (Figure 7 B). Due to the lack of

mature macrophages in the blood, the pSTAT1 staining could not be assessed in those cells. Detection of pSTAT1 in tumour infiltrating myeloid cells failed due to low cell numbers.

We concluded that granulocytes are likely to be dispensable for IL-12-mediated tumour rejection, whereas the involvement of macrophages cannot be excluded.

»8.2 The effects of IFNγ on the tumour vasculature

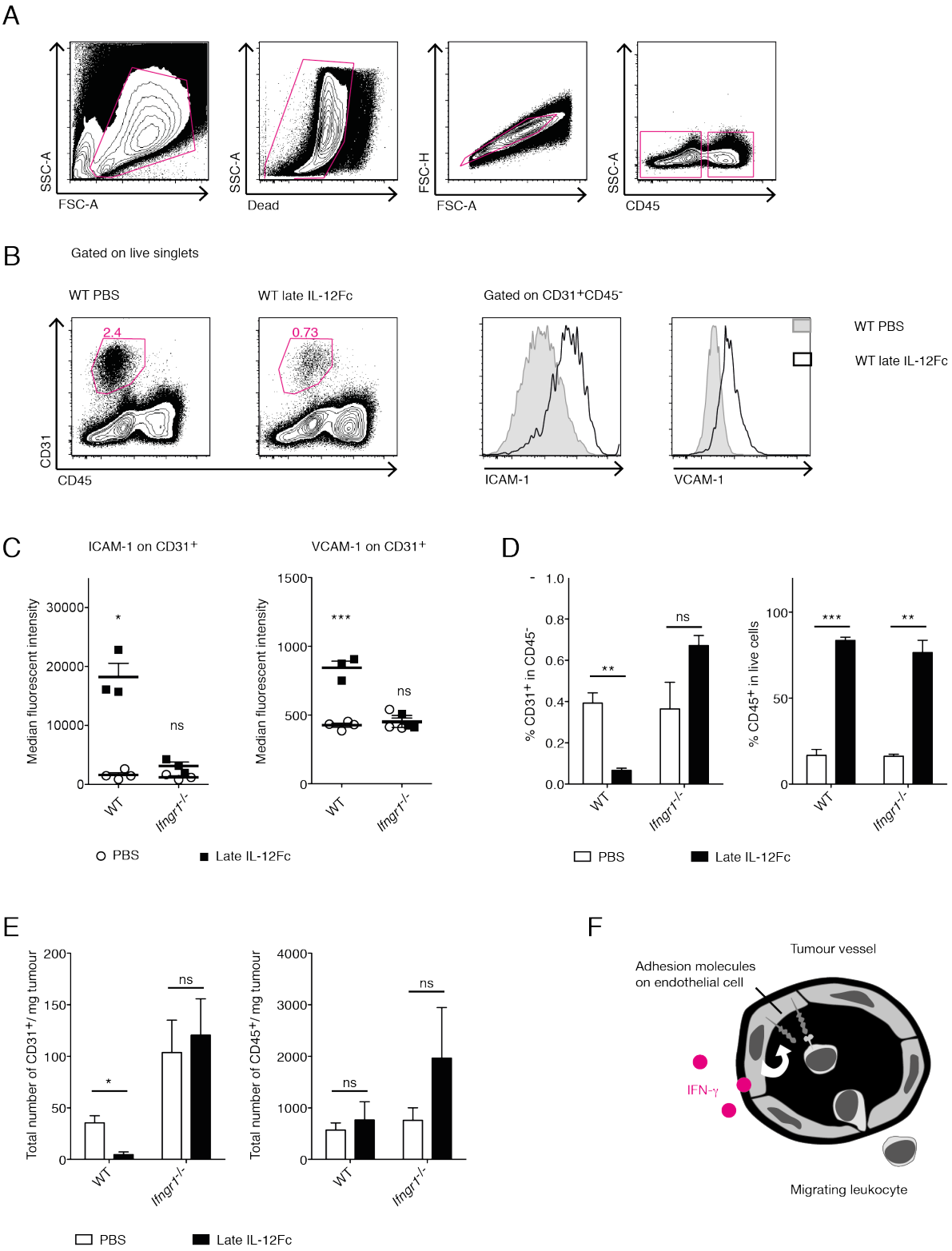
Our data suggested that lymphocytes and monocyte-derived cells were not required to respond to IFNγ to induce IL-12Fc-mediated tumour suppression upon late treatment. As this excluded the majority of immune cell types that accumulated in the tumour tissue upon IL-12 treatment, we focused our further studies on tumour stromal cells. IL-12-induced IFNγ has previously been shown to elicit potent anti-angiogenic effects, inhibiting tumour growth (274). Anti-angiogenic mediators were mostly described to derive from IFNγ responsive leukocytes and tumour cells (189,191), but direct effects of IFNγ on the endothelial cells have also been suggested (188). Moreover, IL-12 administration was previously shown to elicit an up-regulation of ICAM-1 and VCAM-1 on tumour vessels (189,198). This alteration of the adhesion molecule expression on the vasculature may facilitate leukocyte infiltration. Thus the accumulation of immune cells within IL-12Fc treated tumours, observed in this study, further supports a role of the tumour endothelium.

»8.2.1 IL-12-induced IFNγ increases ICAM-1 and VCAM-1 expression and inhibits growth of endothelial cells

The effect of IFNγ on the tumour vasculature was examined in IL-12Fc or PBS administered tumours from WT and *Ifngr1*$^{-/-}$ mice on day 19 of growth (Figure 8 A). Upon isolation of CD31^{+}CD45^{-} endothelial cells from the tumours, expression of the adhesion molecules ICAM-1 and VCAM-1, and endothelial cell numbers were determined by flow cytometry (Figure 8 B).

Figure 8. IL-12Fc treatment leads to IFNγ-dependent alterations of the tumour vasculature. (A) Gating strategy on live population, singlets, and CD45^{-} and CD45^{+} cells within WT tumours. Similar gating approaches were used to analyse endothelial cells isolated from the tumour. (B) Representative FACS plots showing gating on CD31^{+} CD45^{-} events and expression of ICAM-1 and VCAM-1 on these CD31^{+} tumour endothelial cells in late IL-12Fc treated or PBS administered WT tumours. (C) Quantification of median fluorescent intensities for ICAM-1 and VCAM-1 on CD31^{+}CD45^{-} cells for treated or PBS administered tumours grown in *Ifngr1*$^{-/-}$ and WT mice. (D) Frequency of CD31^{+} endothelial cells in CD45^{-} events and of CD45^{+} leukocytes within live, cells in IL-12Fc treated or PBS administered tumours from *Ifngr1*$^{-/-}$ and WT mice. (n=3-4, mean ± S.E.M, experiment performed twice). (E) Total number of CD31^{+} and CD45^{+} cells in treated or PBS administered tumours of *Ifngr1*$^{-/-}$ and WT mice (n=3-4, mean ± S.E.M, experiment performed twice). (F) Illustration of working hypothesis showing the stimulatory effect of IFNγ on adhesion molecule expression on the tumour vasculature, facilitating leukocyte transmigration to the tissue.

Figure 8



This analysis confirmed that IL-12Fc treatment induced a substantial up-regulation of ICAM-1 and VCAM-1 on the tumour vasculature compared to PBS administered controls (Figure 8 B

and C). The increase of ICAM-1 and VCAM-1 expression was highly dependent on IFNγ signalling, as it was abrogated in *Ifngr1*$^{-/-}$ mice (Figure 8 B, C). In late IL-12Fc treated animals the frequency and total numbers of CD31^{+}CD45^{-} cells was markedly reduced in an IFNγ-dependent manner (Figure 8 B, D, E). Similar changes were also detected on day 13 of tumour growth and were, thus, independent of the tumour size (Data not shown). These results suggested, that IFNγ inhibits angiogenesis by its direct or indirect effects on endothelial cells, which may cause the growth regression of IL-12Fc treated tumours. The frequency of infiltrating leukocytes increased upon IL-12Fc treatment in WT and *Ifngr1*$^{-/-}$ mice. (Figure 8 D). As previously demonstrated, the absolute number of CD45^{+} infiltrating leukocytes was comparable in presence and absence of IFNγ signalling at this late time point in tumour growth (Figure 8 E). Thus, on day 19 of tumour progression the leukocyte migration into the tumour was disconnected from the enhanced ICAM-1 and VCAM-1 expression induced by IL-12Fc and IFNγ.

Figure 9

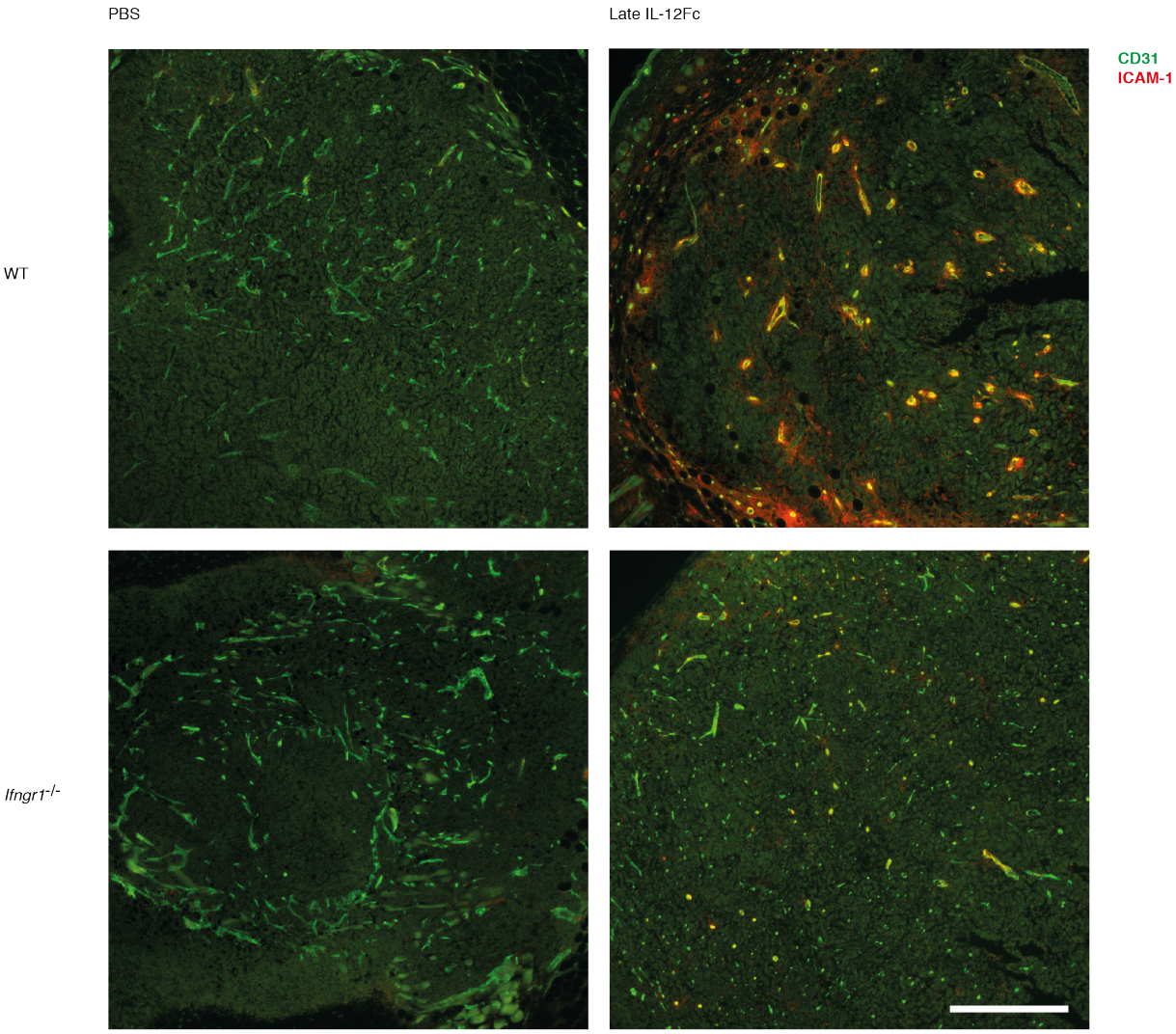


Figure 9. IFNγ-dependent upregulation of ICAM-1 expression on the tumour vasculature upon IL-12Fc treatment assessed by immunofluorescence analysis. Representative graphs showing detection of ICAM-1 (red) and CD31 (green) by immunofluorescent staining in late IL-12Fc treated or PBS administered B16F10 tumours on day 13 of growth in *Ifngr1*$^{-/-}$ and WT mice. (scale bar refers to 500 μm, n=5, experiment performed twice

To confirm these results, immunofluorescent analysis of tumour sections from IL-12Fc or PBS administered WT and *Ifngr1*$^{-/-}$ mice was performed. Only IL-12Fc treated WT mice showed a strong ICAM-1 signal on CD31^{+} tumour vessels, supporting the essential role of IFNγ for the induction of this adhesion molecule (Figure 9). A decreased number of small vessels were observed in IL-12Fc treated tumours of WT mice although this was not quantified (Figure 9). This would further support the evidence of anti-angiogenic mechanisms provoked by IL-12Fc

Thus, IFNγ seems to regulate tumour vessel formation and adhesion molecule expression in IL-12Fc treated tumours.

»8.2.2 IFNγ signalling on endothelial cells regulates ICAM-1 and VCAM-1 expression *in vitro*.

In order to distinguish direct from indirect effects of IFNγ on endothelial cells an *in vitro* assay was utilised. Cells from the MS-1 (MILE SVEN 1) pancreas-derived endothelial cell line were cultured in the presence or absence of cytokines and/or purified lymphocytes. After culture for 48 hours the MS-1 cells were harvested and analysed by flow cytometry. Treatment with IL-12Fc alone showed no effect on ICAM-1 and VCAM-1 expression on MS-1 cells, while IFNγ induced a significant up-regulation of both adhesion molecules (Figure 10 A). Purified RORγt^{fm+} ILCs or NK1.1^{+} NK cells stimulated with IL-12Fc provoked elevated adhesion molecule levels on MS-1 cells. Blocking IFNγ by an anti-IFNγ antibody led to a complete ablation of the adhesion molecule up-regulation induced by the IL-12-treated lymphocytes (Figure 10 A). Labelling of MS-1 cells with a fluorescent dye allowed the quantification of cell proliferation. Interestingly, IFNγ increased MS-1 proliferation rates, while all other conditions had no impact (Figure 10 B).

These results suggest that direct IFNγ signalling may regulate adhesion molecule expression on endothelial cells.

Figure 10

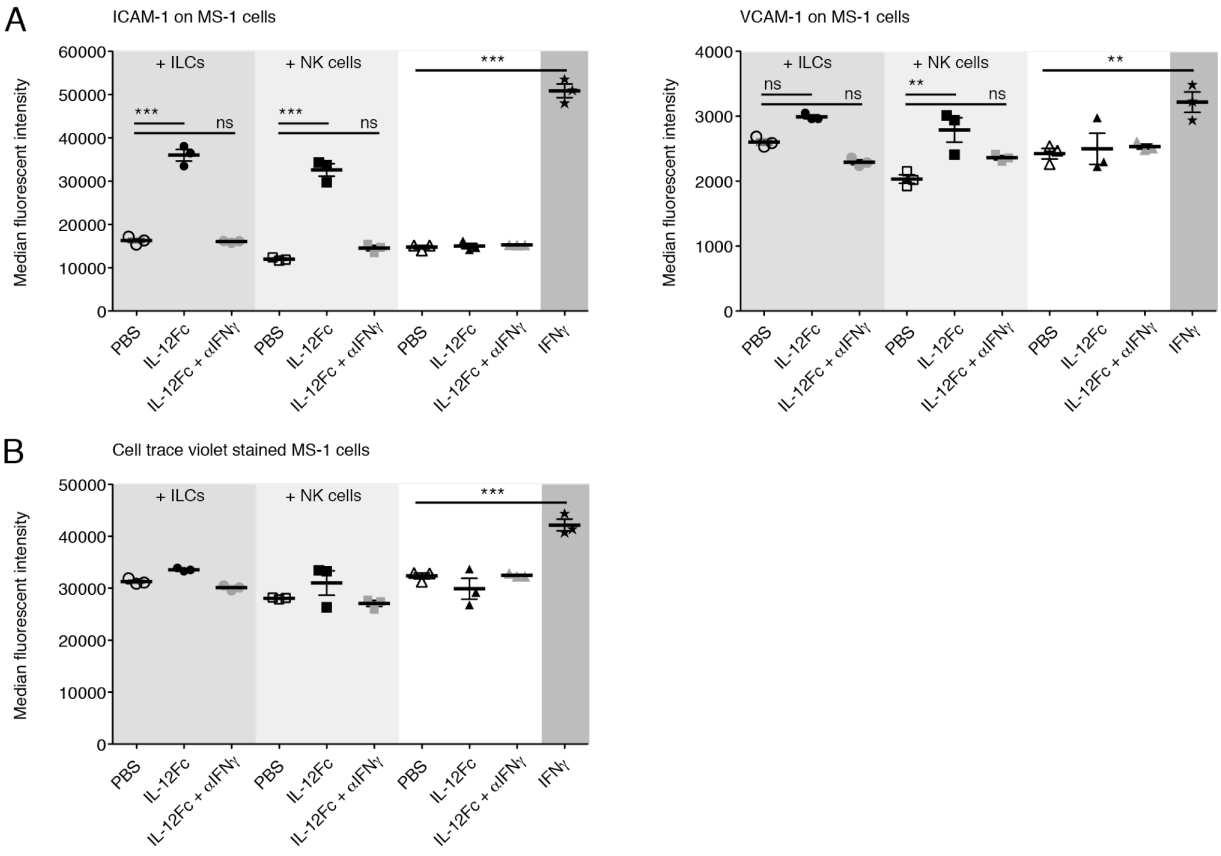


Figure 10. IFNγ secreted by lymphocyte populations directly effects the adhesion molecule expression on the MS-1 endothelial cell line. (A) Median fluorescent intensity of ICAM-1 (left) and VCAM-1 (right) on MS-1 cells co-cultured with RORγt^{fm+} ILCs and fm^{-}NK1.1^{+} NK cells FACS purified from *Rorc*$^{fm+}$*Rag1*$^{-/-}$ mice upon *in vitro* addition of IL-12Fc alone or in combination with a blocking anti-IFNγ antibody compared to controls. IFNγ supplementation was used as control. (B) MS-1 proliferation determined by using the dye cell trace violet under the conditions described in A. (n=3, mean ± S.E.M, experiment performed twice)

»8.2.3 Direct IFNγ signalling causes endothelial cell alterations but is dispensable for IL-12Fc-induced tumour suppression *in vivo*.

In vivo, endothelial cells were targeted by utilising mice that express the Cre recombinase under the endothelium specific VE-Cadherin promoter (*VEC*-Cre) (275). VE-Cadherin is a transmembrane protein involved in homotypic cell adhesion, which is continuously expressed by differentiated quiescent endothelial cells (275-277). Notably, VE-Cadherin is also transcribed in hematopoetic stem cells with endothelial origin, which are targeted in *VEC*-Cre mice (275,278). Using a similar approach as for the LysM-specific ablation of IFNγ signalling, we bred *VEC*-Cre^{+}*Ifngr*$^{fl/fl}$ mice to disrupt IFNγ receptor expression on endothelial cells.

Figure 11

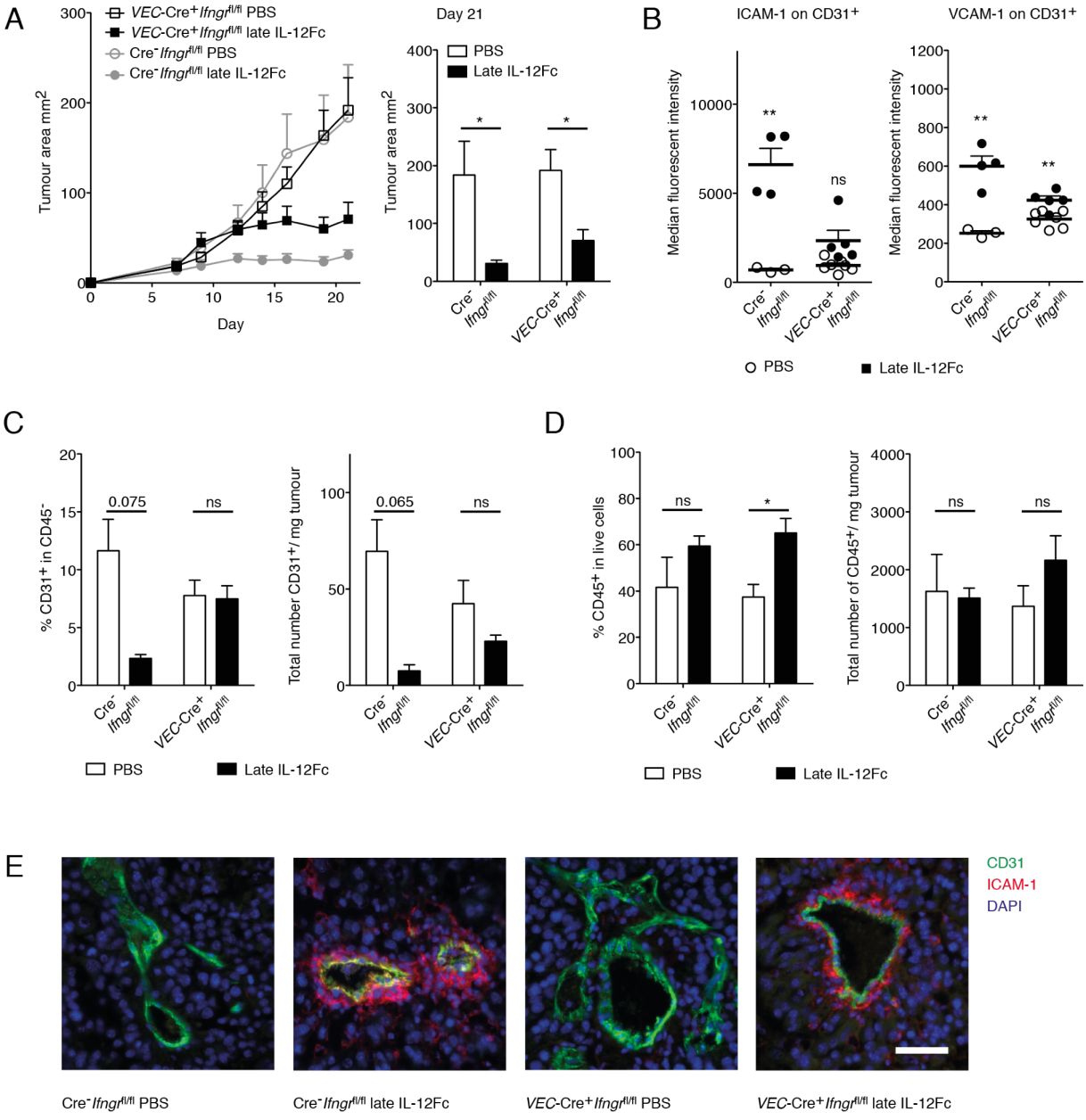


Figure 11. Alteration of tumour vasculature upon late IL-12Fc treatment mainly relies on the direct impact of IFNγ on the endothelium. (A) Growth curve and size on day 21 of tumours collected from *VEC*-Cre^{+}*Ifngr1*$^{fl/fl}$ mice and Cre^{-}*Ifngr1*$^{fl/fl}$ littermates upon late IL-12Fc treatment compared to PBS administered controls (pooled data from two independent experiments, n=7-11, mean ± S.E.M) (B) Median fluorescent intensity of ICAM-1 and VCAM-1 on CD31^{+}CD45^{-} cells from tumours of *VEC*-Cre^{+}*Ifngr1*$^{fl/fl}$ and Cre^{-}*Ifngr1*$^{fl/fl}$ mice treated with IL-12Fc or PBS administered. (C) Frequency and absolute numbers of the CD31^{+} population in CD45^{-} cells within treated and control tumours in *VEC*-Cre^{+}*Ifngr1*$^{fl/fl}$ and Cre^{-}*Ifngr1*$^{fl/fl}$ mice. (D) Frequency of live CD45^{+} cells and total numbers within treated or PBS administered tumours in *VEC*-Cre^{+}*Ifngr1*$^{fl/fl}$ and Cre^{-}*Ifngr1*$^{fl/fl}$ mice. (n=3-6, mean ± S.E.M) (E) Representative immunofluorescent images showing ICAM-1 (red) and CD31 (green) and DAPI (blue) in treated and control tumours on day 21 of growth from *VEC*-Cre^{+}*Ifngr1*$^{fl/fl}$ and Cre^{-}*Ifngr1*$^{fl/fl}$ mice (scale bar refers to 50 μm). (n=5, experiments performed twice)

VEC-Cre^{+}*Ifngr*$^{fl/fl}$ mice and Cre^{-}*Ifngr*$^{fl/fl}$ littermates were challenged with B16F10 tumours and the IL-12Fc-induced tumour suppression was compared. Tumour growth upon IL-12Fc treatment was similar in *VEC*-Cre^{+}*Ifngr*$^{fl/fl}$ and Cre^{-}*Ifngr*$^{fl/fl}$ animals (Figure 11 A). In tumours from Cre^{-}*Ifngr*$^{fl/fl}$ mice ICAM-1 and VCAM-1 were upregulated upon IL-12Fc treatment, whereas this effect was largely abolished *VEC*-Cre^{+}*Ifngr*$^{fl/fl}$ mice (Figure 11 B). Frequency and absolute numbers of CD31^{+}CD45^{-} endothelial cells were reduced in Cre^{-}*Ifngr*$^{fl/fl}$ but not in *VEC*-Cre^{+}*Ifngr*$^{fl/fl}$ mice, although statistical testing did not reach significance (Figure 11 C). The accumulation of immune cells in the tumour tissue was not influenced by the mouse genotype (Figure 11 D). Thereby, the endothelium in *VEC*-Cre^{+}*Ifngr*$^{fl/fl}$ mice underwent similar changes as observed in *Ifngr1*$^{-/-}$ mice. This implied that recombination upon Cre expression in endothelial cells was successful in *VEC*-Cre^{+}*Ifngr*$^{fl/fl}$ mice. In contrast, immunofluorescent detection of ICAM-1 and CD31 in tumour sections showed an increase of ICAM-1 signal on IL-12Fc treated tumours from both Cre^{-}*Ifngr*$^{fl/fl}$ and *VEC*-Cre^{+}*Ifngr*$^{fl/fl}$ mice. In tumours from *VEC*-Cre^{+}*Ifngr*$^{fl/fl}$ compared to Cre^{-}*Ifngr*$^{fl/fl}$ mice the ICAM-1 staining was observed to be surrounding tumour vessels rather than locating on the cell surface of CD31^{+} cells. ICAM-1 expression on IFNγ responsive tumour cells and leukocytes in the tumour may have influenced this result.

Taken together, we conclude that the direct effect of IFNγ on the tumour vasculature does not influence tumour growth upon late treatment with IL-12Fc.

»9 Cellular responses to early IL-12Fc treatment of s.c. B16F10 tumours

Figure 12

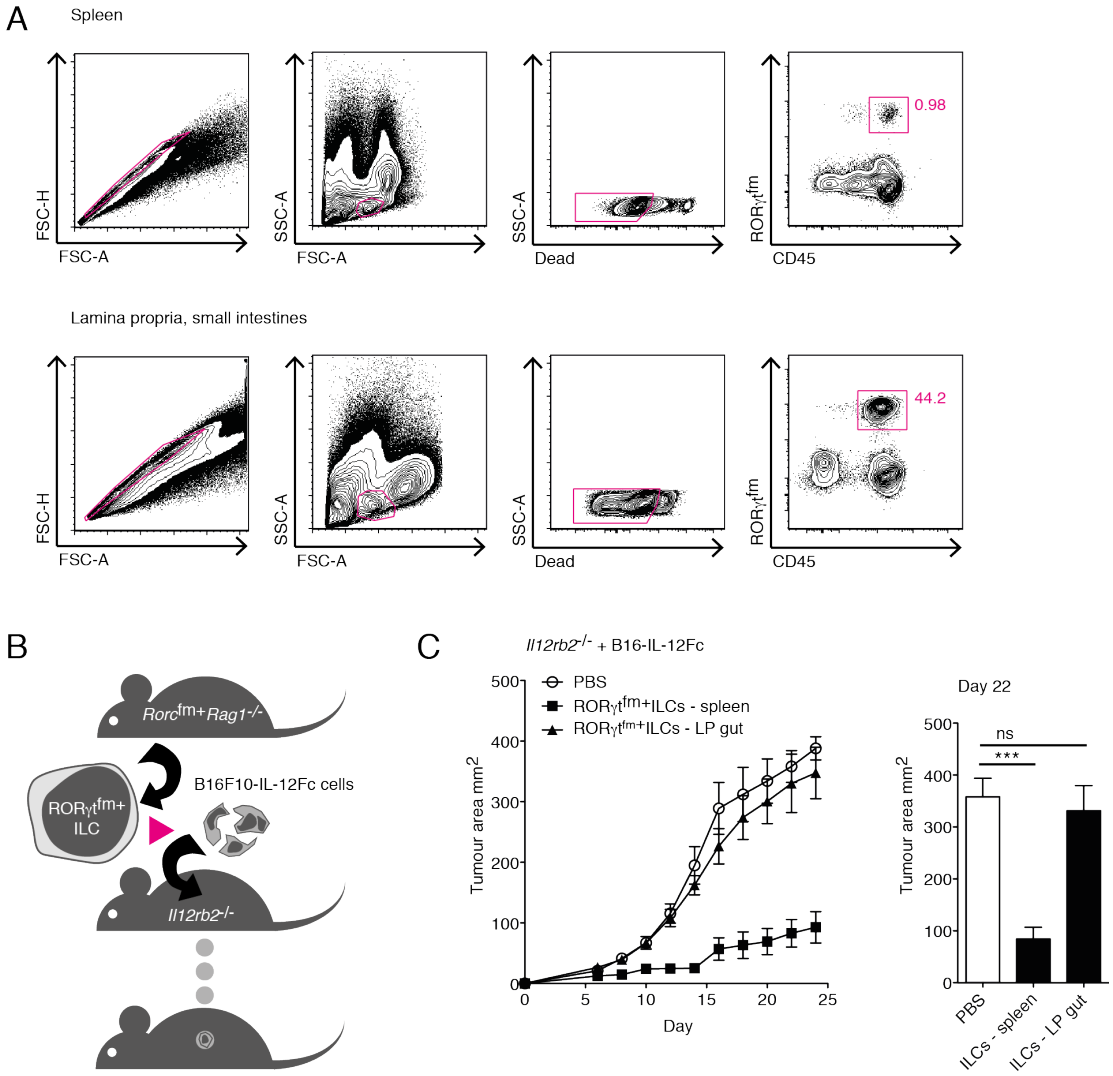


Figure 12. IL-12Fc responsive splenic but not gut RORγt-dependent ILCs are sufficient to induce tumour suppression upon early IL-12Fc treatment. (A) Gating strategy for singlets, lymphocytes, live events and CD45^{+} RORγt^{fm+} cells from spleen and the lamina propria of the small intestines (from here on referred to as LP gut) in *Rorc*$^{fm+}$*Rag1*$^{-/-}$ mice. (B) Illustration of the experimental setup for the purification of RORγt^{fm+} ILCs form *Rorc*$^{fm+}$*Rag1*$^{-/-}$ donors and their co-transfer together with B16F10-IL-12Fc cells into *Il12rb2*$^{-/-}$ recipients. (C) Growth of B16F10-IL-12Fc tumours in *Il12rb2*$^{-/-}$ mice upon transfer of 6'000-10'000 RORγt^{fm+} ILCs derived from spleen or LP gut, or PBS and correspondent tumour sizes on day 22 post inoculation. (n=4-5, mean ± S.E.M, experiment performed 3 times. Cell purification was performed by K. Nussbaum and S. Burkhard; K. Nussbaum measured the tumours)

»9.1 Splenic, but not gut-derived RORγt-dependent ILCs induce the anti-tumour response upon early IL-12Fc treatment

RORγt-dependent ILCs derived from the spleen have previously been described to induce the suppression of tumour growth upon stimulation with IL-12Fc (198). Moreover, the immune system of the gut harbours a large population of RORγt-dependent ILCs (50). Thus, we aimed to investigate whether these cells shared the tumour suppressive potential with their splenic counterpart. RORγt^{fm+} ILCs in the lamina propria of the small intestines (referred to as gut ILCs) and from the spleen of *Rorc*$^{fm+}$*Rag1*$^{-/-}$ mice were identified by their expression of eYFP and CD45, and FACS purified (Figure 12 A). Similar to previous experiments (198), we co-injected RORγt^{fm+} ILCs together with B16F10-IL-12Fc into *Il12rb2*$^{-/-}$ mice (Figure 12 B). Upon transfer, splenic RORγt^{fm+} ILCs efficiently controlled tumour growth, whereas gut-derived ILCs failed to mount this response (Figure 12 C).

This data shows that there are functional differences between RORγt^{fm+} ILCs in the gut and spleen. We hypothesised that their diverging function could be explained by the presence of different ILC subsets or developmental stages in these two organs.

»9.2 RORγt^{fm+} ILCs from the tumour, spleen and gut differ regarding their expression of surface markers

In order to investigate the differences between gut- and spleen-derived ILCs we first analysed their phenotype. The population of RORγt^{fm+} ILCs in gut and spleen were characterised by surface markers that have previously been used to scrutinise ILC subsets. The expression pattern observed on RORγt^{fm+} ILCs was compared to fm^{-}CD49b^{+}NK1.1^{+} NK cells from the spleen (Figure 13 B). NK cell markers such as NKp46, CD49b and NK1.1 were expressed at similar levels on both splenic ILCs and NK cells, whereas gut ILCs expressed lower levels (Figure 13 A). In the case of ILC characteristic surface markers, such as Thy-1, IL-7Rα and cKit, expression levels were lower on splenic ILCs and NK cells compared to gut RORγt^{fm+} ILCs. Sca-1 expression was similar in all populations (Figure 13 A). In conclusion, splenic ILCs mirrored the expression pattern of NK cells whereas gut RORγt^{fm+} ILCs clearly diverged phenotypically. This was supported by gene array data, further illustrating transcriptional differences between RORγt^{fm+} ILCs from gut and spleen (Data not shown).

Figure 13

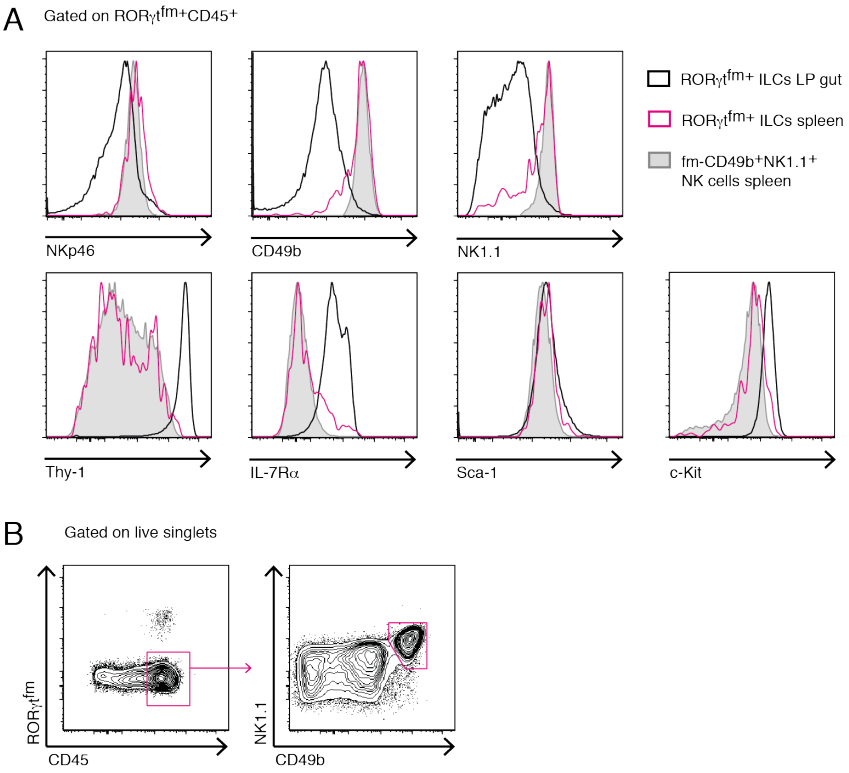


Figure 13. RORγt^{fm+} ILCs from spleen and gut differ in their surface marker expression. (A) Representative histograms showing surface expression levels of NK cell markers (NKp46, CD49b and NK1.1) and classical ILC markers (Thy-1, IL-7ra, Sca-1 and c-Kit) on splenic and LP gut RORγt^{fm+} cells and fm^{-} CD49b^{+}NK1.1^{+} cells isolated from *Rorc*$^{fm+}$*Rag1*$^{-/-}$ mice. (B) Gating on CD49^{+}NK1.1^{+} cells within the fm^{-}CD45^{+} compartment. (n=3, mean ± S.E.M, experiment was performed 6 times, depicted data by S. Burkhard)

Thus, we sought to investigate the expression of surface markers on RORγt^{fm+} ILCs within the tumour tissue. For this purpose *Rorc*$^{fm+}$*Rag1*$^{-/-}$ mice were challenged with B16F10 tumours, which were administered with IL-12Fc form day 0 onwards. We investigated early IL-12Fc treated tumours on different days after B16F10 inoculation and found that RORγt^{fm+} ILC density was highest on day 2 and 4 of growth (Data not shown). Thus, tumours and spleens from IL-12Fc or PBS administered mice were further compared for the presence of these cells on day 4-post inoculation, (Figure 14 A). Similar to their splenic counterpart, the majority of intratumoural RORγt^{fm+} ILCs expressed CD49b and NKp46 (Figure 14 A, B). In contrast, RORγt^{fm+} cells from the tumour displayed a uniform high expression for Thy-1, whereas splenic cells expressed variable levels (Figure 14 B). Early IL-12Fc treatment increased the frequency of RORγt^{fm+} ILCs within the tumour infiltrating CD45^{+} leukocytes, while phenotypical characteristics did not change (Data not shown).

Total numbers of intratumoural ILCs were not elevated (Figure 14 C), which may be explained by the reduced tumour size in IL-12Fc treated mice. Due to small tumour masses on day 4, diameters of the lesions and tissue weight could not be determined accurately. In the spleen IL-12Fc treatment led to both elevated frequency and total numbers of RORγt^{fm+} ILCs (Figure 14 C). However, the enlargement of the spleen upon IL-12Fc treatment should be taken into account.

In summary, these data suggest that tumour infiltrating RORγt^{fm+} ILCs share similarities with both spleen- and gut-derived ILCs.

Figure 14

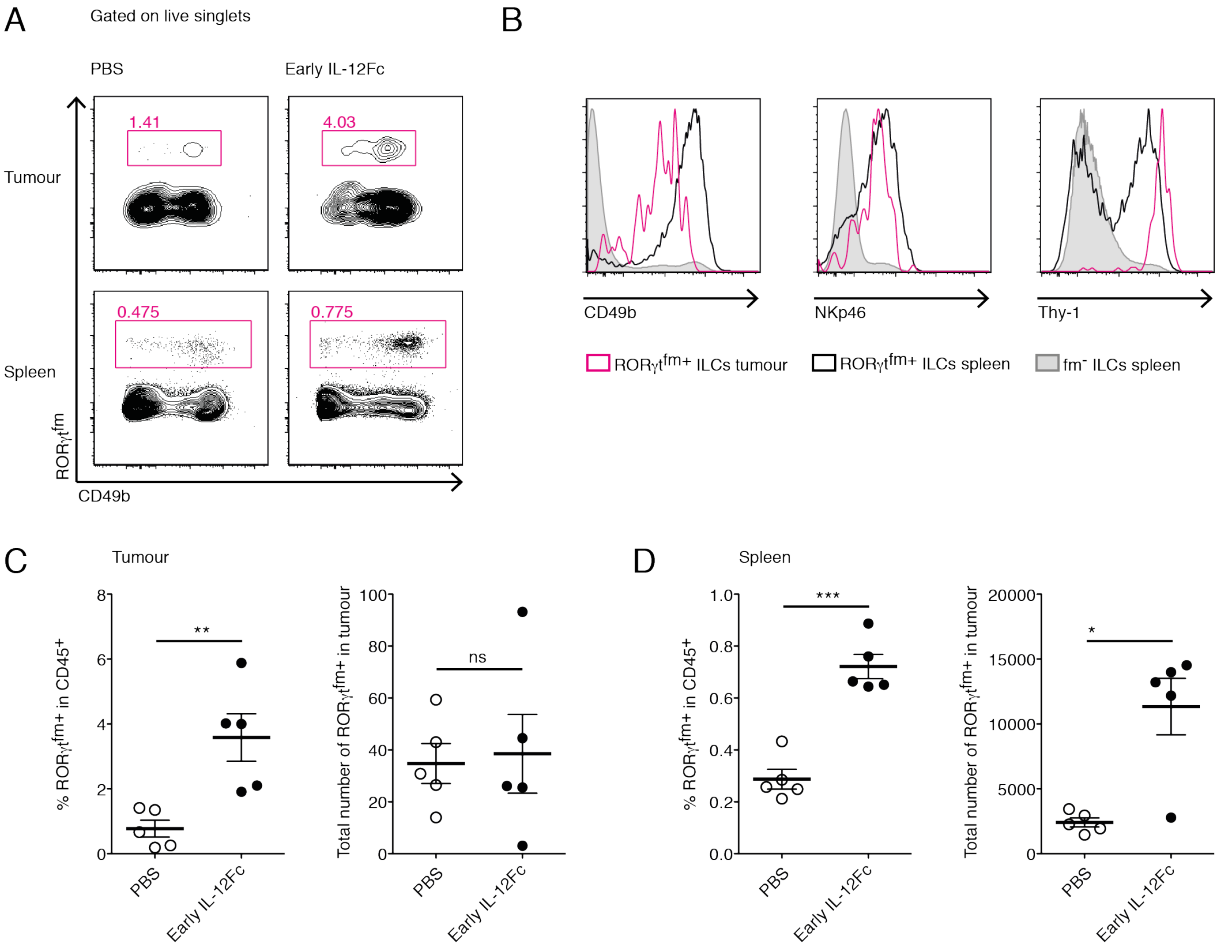


Figure 14. RORγt^{fm+} ILCs infiltrate the IL-12Fc-treated tumour at early time points and share similarities with both their splenic and gut counterpart. (A) Gating on RORγt^{fm+} CD45^{+} live cells isolated from spleens and tumours *Rorc*$^{fm+}$*Rag1*$^{-/-}$ mice, upon early IL-12Fc or PBS administration. (B) Representative histograms showing expression of CD49b, NKp46 and Thy-1 on RORγt^{fm+} ILCs and fm^{-} NK cells in tumour and spleen. (C) Frequency and total numbers of RORγt^{fm+} cells within the CD45^{+} compartment in IL-12Fc treated or PBS administered tumours. (D) Frequency and total numbers of RORγt^{fm+} events within the CD45^{+} compartment in the spleen of treated or PBS administered mice. (n=5, mean ± S.E.M, experiment was performed twice by S. Burkhard)

»9.3 Splenic and gut RORγt^{fm+} ILCs differ in transcriptional levels of IL-12Rβ2 and IL-23R.

Next, we investigated whether the discrepancy in the anti-tumour function of splenic and gut ILCs could be explained by different responsiveness to IL-12Fc. RORγt^{fm+} ILCs from the gut and spleen were purified and cultured in presence or absence of IL-12Fc for 12 hours. Thereafter, transcriptional levels of IL-12Rβ2 and IL-23R receptor subunits were quantified by qPCR. Notably, IL-12Rβ2 expression was approximately 5 fold higher in splenic compared to gut RORγt^{fm+} ILCs (Figure 15 A). In contrast, the transcription level for IL-23R was elevated on gut compared to splenic ILCs, although this was not observed to be statistically significant (Figure 15 B). Due to low RORγt^{fm+} ILCs numbers obtained from the spleen, the relative expression values varied substantially, but always showed the same trends. Stimulation of purified RORγt^{fm+} ILCs with IL-12Fc *in vitro* increased mRNA levels for IL-12Rβ2 on both ILC populations (Figure 15 C). Moreover, IFNγ levels in the supernatants were elevated upon IL-12Fc exposure of splenic compared to gut RORγt^{fm+} ILCs (Data not shown).

The observed differences in IL-12Rβ2 expression imply that RORγt^{fm+} ILCs form the spleen are more responsive to IL-12 compared to gut ILCs. This may explain why only splenic cells could reject IL-12Fc secreting tumours in the transfer experiment.

Figure 15

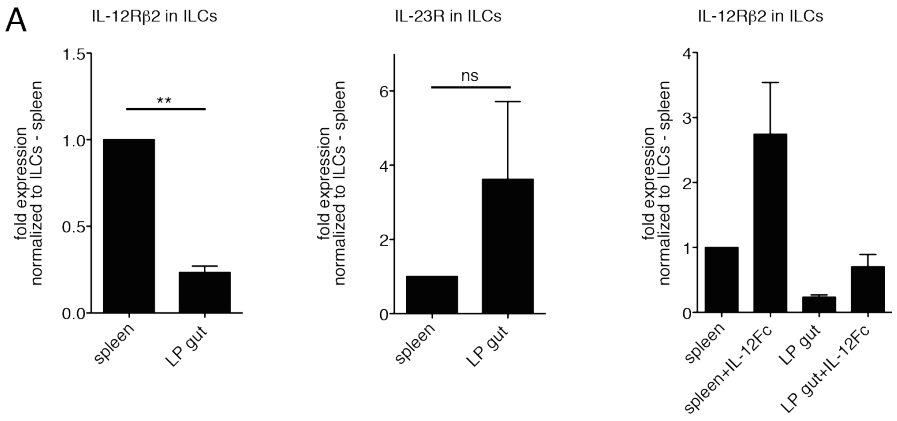


Figure 15. Splenic RORγt^{fm+} ILCs express higher levels of IL-12rβ2 compared to gut ILCs. (A) IL-12rb2 (left) and IL-23r (right) expression levels in FACS purified splenic and LP gut RORγt^{fm+} ILCs , assessed by quantitative PCR. (C) IL-12rb2 expression levels upon *in vitro* stimulation with IL-12Fc for 12 hours of RORγt^{fm+} ILCs derived from spleen and LP gut. (mean ± S.E.M, pooled data from 3 individual experiments with 3 technical replicates each, statistical significance was assessed by a paired T test. Purification of cells was performed by K. Nussbaum and S. Burkhard. Isolation of mRNA and quantification of expression levels was performed by S. Burkhard)

»9.4 RORγt^{fm+} ILCs in the spleen down-regulate RORγt and adopt ILC1 characteristics

RORγt-dependent ILC3s are reported to adopt phenotypical and functional features of ILC1s, such as expression of NK cell markers and the production of IFNγ, which coincide with the loss of RORγt expression (239,240). More recently, this differentiation was shown to be induced upon IL-12 and IL-18 stimulation (240,241). As splenic RORγt^{fm+} ILCs clearly displayed characteristics of ILC1s, we investigated the RORγt expression by splenic and gut-derived cells. These populations were analysed in *Rorc*$^{rep+}$*Rag1*$^{-/-}$ mice, in which RORγt expressing ILCs were labelled with eGFP (RORγt^{rep+}), and compared to RORγt^{fm+} cells. While in the gut a population of RORγt^{rep+} was detected, in the spleen nearly all cells from the *Rorc*$^{rep+}$*Rag1*$^{-/-}$ reporter mice were eGFP negative (Figure 16 A). This suggested that some RORγt^{fm+} ILCs from the gut actively expressed RORγt, while most splenic RORγt^{fm+} ILCs had down-regulated RORγt. The expression of the transcription factors RORγt and T-bet in RORγt^{fm+} ILCs was further assessed by intracellular staining and compared to CD49b^{+} NK cells and CD4^{+} T_H cells. Whereas the vast majority of gut RORγt^{fm+} ILCs expressed RORγt, the majority of the splenic compartment was RORγt^{-}. Instead, RORγt^{fm+} ILCs from the spleen and NK cells expressed T-bet, while in the gut both T-bet^{+} and T-bet^{-} cells were detected (Figure 16 B). Approximately 20% of splenic RORγt^{fm+} ILCs were shown to secrete IFNγ, while only 2.5% of gut-derived cells expressed this cytokine. The IL-17A secretion was observed to be low in both RORγt^{fm+} ILC populations in naïve mice (Figure 16 C).

This data demonstrates that RORγt^{fm+} ILCs in the spleen have down-regulated RORγt and express T-bet. Thus, we assume that these cells have undergone the transition from ILC3 to ILC1 function, which allows them to secrete IFNγ and possibly supplies them with their anti-tumour properties.

Figure 16

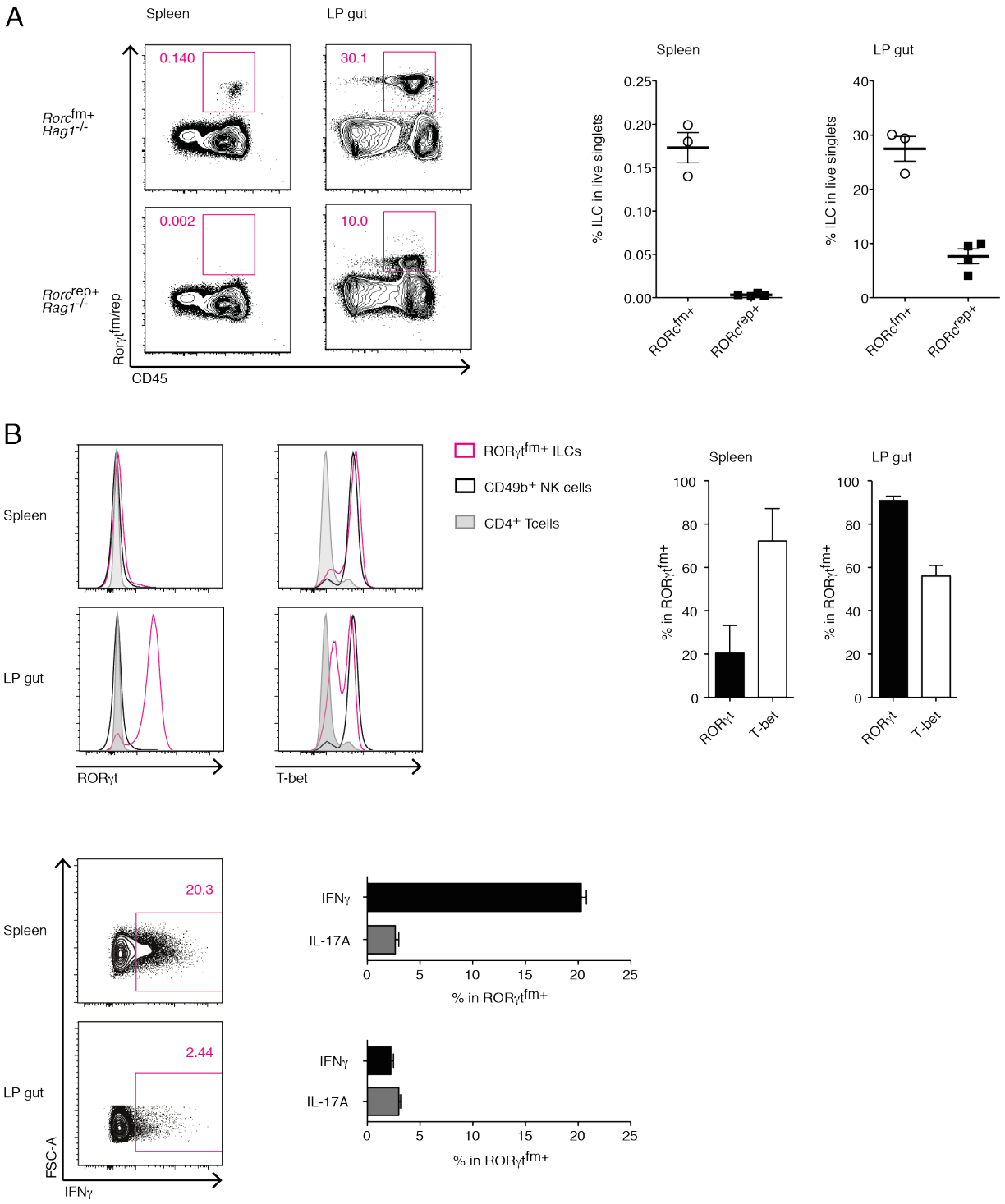


Figure 16. Splenic RORγt^{fm+} are have down-regulated their RORγt expression and thereby adopt ILC1 characteristics. (A) Left: Splenic and LP gut RORγt^{fm+} and RORγt^{rep+} ILCs isolated from *Rorc*$^{fm+}$*Rag1*$^{-/-}$ and *Rorc*$^{rep+}$*Rag1*$^{-/-}$ mice, respectively. Right: Frequency of RORγt^{fm+} and RORγt^{rep+} events in spleen and LP gut. (n=3-4, mean ± S.E.M performed 3 times, depicted data by S. Burkhard) (B) Top row left: Representative histograms of RORγt and T-bet expression, assessed by intracellular staining of purified RORγt^{fm+} ILCs , CD49+ NK cells and CD4+ T cells form spleen and LP gut. Top row right: frequencies of RORγt and T-bet expressing splenic and LP gut-derived RORγt^{fm+} ILCs. Bottom row: FACS plots showing IFNγ secretion and frequencies of IFNγ and IL-17A expressing RORγt^{fm+} ILCs in spleen and LP gut (mean ± S.E.M, experiment performed twice by K. Nussbaum).

»9.5 The anti-tumour response upon early IL-12Fc administration does not depend on IL-15 signalling

NCR$^+$ ILCs were recently demonstrated to depend on IL-15 for their development or maintenance, as their population was found to be decreased in the gut of IL-15-deficient mice. More specifically, the numbers of ILC1s and ILC3s, expressing the natural cytotoxicity receptors NKp46 and NK1.1, were shown to be reduced (234). Thus, we aimed to assess the role of IL-15 signalling on the maintenance of the splenic pool of ILCs and the anti-tumour response upon early IL-12Fc treatment by utilising IL-15 receptor-deficient mice (*Il15ra*$^{-/-}$).

Figure 17

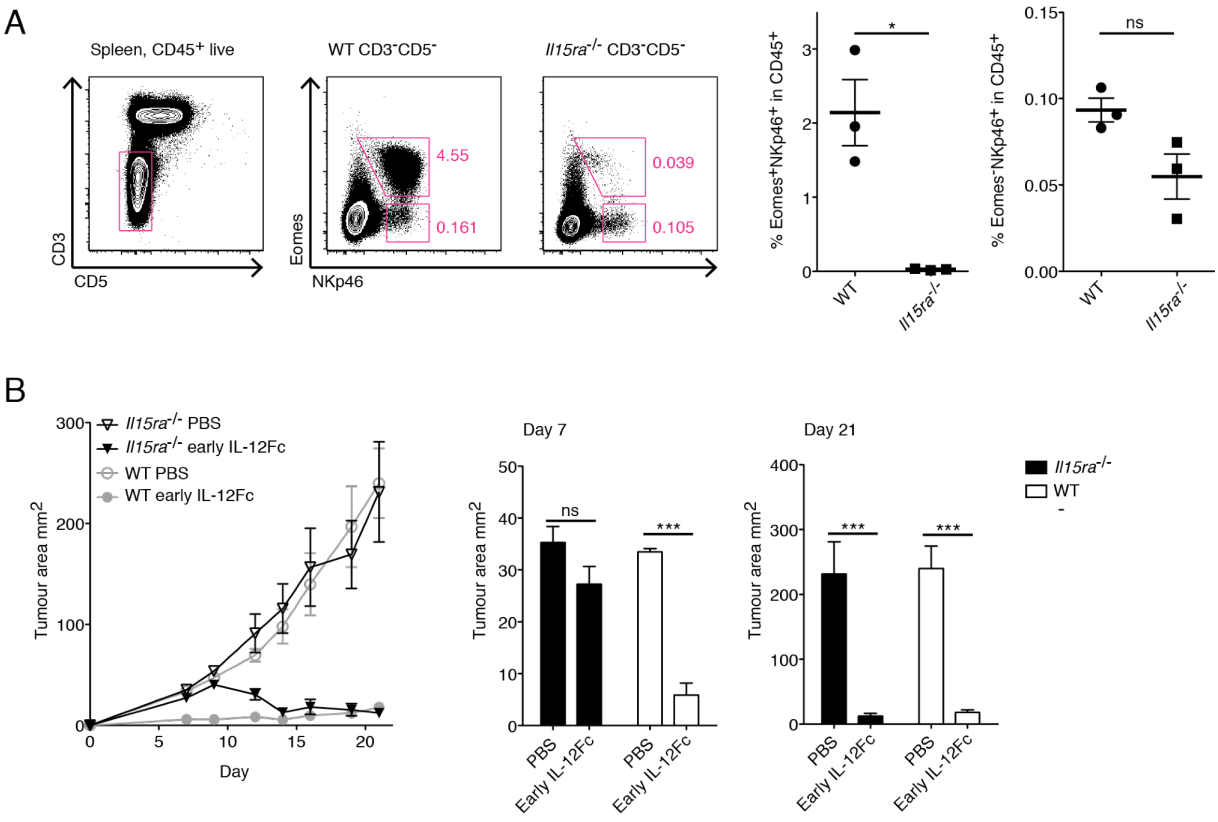


Figure 17. The effect of early IL-12Fc treatment on the tumour growth is independent of IL-15 signalling. (A) Left: Gating on Eomes positive and negative NKp46$^+$CD3$^-$CD5$^-$ cells in the spleen of naïve WT and *Il15ra*$^{-/-}$ mice. Right: Quantification of the frequencies Eomes$^+$ and Eomes$^-$ cells defined in the FACS plots (left). (n=3, mean ± S.E.M, experiment performed once). (B) Left: Tumour growth in *Il15ra*$^{-/-}$ and WT mice upon early IL-12Fc treatment or PBS administration. Right: Tumour sizes were quantified on day 7- and day 21-post inoculation (n=5, mean ± S.E.M, experiment performed twice).

NKp46^{+}CD3^{-}CD5^{-}CD45^{+} lymphocytes were isolated from the spleen and further distinguished by their expression of Eomes (Figure 17 A). Comparison of the Eomes^{+}NKp46^{+} population in the spleen of *Il15ra*$^{-/-}$ and WT mice revealed a dramatic decrease in frequency of the Eomes expressing population. In contrast, the pool of Eomes^{-}NKp46^{+} cells in was only marginally reduced in *Il15ra*$^{-/-}$ compared to WT mice, although statistical values did not reach significance (Figure 17 A). B16F10 cells were inoculated to *Il15ra*$^{-/-}$ mice and tumour growth was compared to WT controls. *Il15ra*$^{-/-}$ mice showed no impairment in tumour control in comparison to WT mice upon early treatment with IL-12Fc (Figure 17 B). A preliminary experiment further indicated that the majority of RORγt^{fm+} ILCs in the spleen expressed the transcription factor Eomes (Observation by K. Nussbaum not shown).

Thus, tumours were still rejected in absence of IL-15 signalling and thereby Eomes^{+} NK cells and ILCs. We therefore hypothesised that such ILCs may not be necessary for the IL-12Fc-induced tumour suppression.

» 9.6 RORγt^{fm+} ILCs are not necessary for the rejection of early IL-12Fc treated tumours

Similar the previous study by our group (198), we have demonstrated that the response of splenic RORγt^{fm+} ILCs to IL-12Fc is sufficient to induce the subsequent anti-tumour immunity. Further, we aimed to establish whether RORγt-dependent ILCs are also necessary for the rejection of tumours. To address this, we compared tumour growth of early IL-12Fc treated or PBS administered tumours in *Rorc*$^{-/-}$*Rag1*$^{-/-}$ animals to those in *Rag1*$^{-/-}$ mice. Interestingly, the anti-tumour response in *Rorc*$^{-/-}$*Rag1*$^{-/-}$ mice was unaffected by the absence of RORγt-dependent ILCs (Figure 18 A). To further support this data *Rorc*$^{-/-}$*Rag1*$^{-/-}$ splenocytes were co-transferred with B16F10-IL-12Fc to *Il12rb2*$^{-/-}$ mice and compared with splenic cells derived from *Rag1*$^{-/-}$ mice in a preliminary experiment. Both *Rorc*$^{-/-}$*Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ splenocytes were equally efficient at suppressing the tumour growth (Figure 18 B).

This suggests that RORγt^{fm+} ILCs are not necessary for the IL-12Fc-induced anti-tumour response. This implies that other cell populations harbour similar tumour suppressive effects.

Figure 18

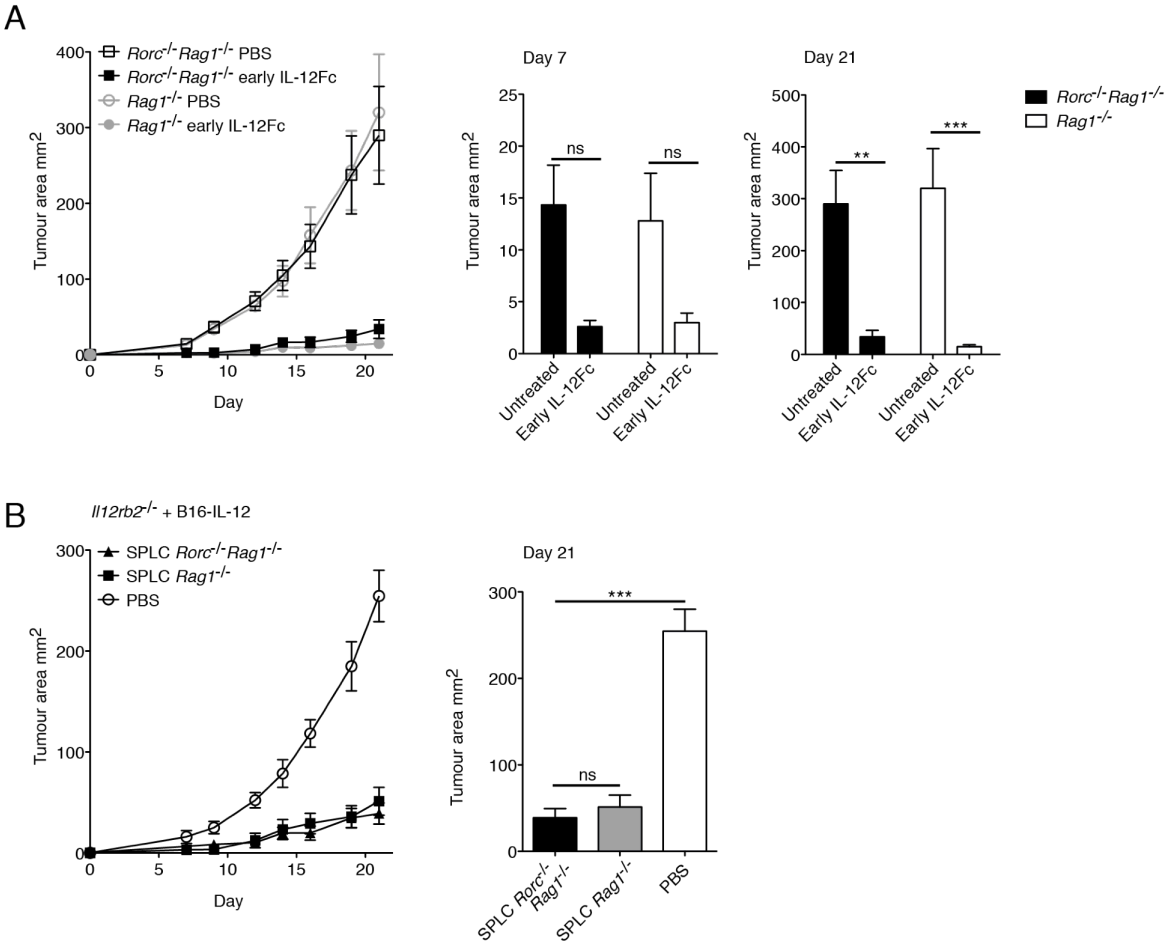


Figure 18. RORγt-dependent ILCs are not necessary for early IL-12Fc-induced anti-tumour responses. (A) Left: Growth of early IL-12Fc treated or PBS administered B16F10 tumours in *Rorc*$^{-/-}$*Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ mice. Right: Quantification of the tumour size on day 7 and day 21 of tumours corresponding to the left graph. (n = 4-5, mean ± S.E.M, experiment performed twice) (B) Left: Tumour growth in *Il12rb2*$^{-/-}$ mice upon transfer of 2 x 10^5 splenocytes (SPLC) isolated from *Rorc*$^{-/-}$*Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ mice together with B16F10-IL-12Fc tumour cells. Right: Quantification of tumour area on day 21 of tumours depicted in left graph. (n=4-5, mean ± S.E.M, experiment performed once)

VII Discussion

»10 Distinct involvement of IFNγ and immune cells upon early and late IL-12Fc treatment

The potent anti-tumour effects elicited by IL-12 treatment have been demonstrated in various experimental models, and raised expectations for the immunotherapy of human cancers. The occurrence of severe side effects upon systemic IL-12 treatment of cancer patients, however, frustrated these hopes. In contrast, several clinical studies reported encouraging effects of IL-12 on human malignancies (206-208,279). Thus, the uncertainty remains, whether modern medicine can benefit from the treatment potential of IL-12 and avoid adverse effects. Many research groups have focused on a more localised and thereby safer delivery of IL-12 to the tumour tissue (206). Understanding the mechanistic principles of IL-12-induced tumour suppression and side effects may further support the development of successful cancer therapies. Molecules, such as cytokines, involved in the IL-12-mediated tumour suppression may themselves be implemented as anti-cancer agents. Furthermore, the function of tumour suppressing cell types in this context could be supported by other immune modulating components. Unfortunately, many studies dedicated to the characterisation of this mechanism presented conflicting results. These discrepancies may be explained by the timing and dosage of IL-12 administration (177), as well as the anatomical location of the tumour (196).

In order to investigate the time dependency of IL-12 treatment we compared two different treatment regimens with IL-12Fc. While both treatment modalities elicited strong anti-tumour effects, different mechanisms of tumour rejection regarding the involvement of IFNγ were observed. What causes this discrepancy that is solely influenced by the timing of IL-12Fc treatment can only be speculated. IL-12Fc transferred simultaneously with B16F10 cells may, for instance, alter or delay the formation of the tumour mass. In this respect, one could investigate whether IL-12Fc can also inhibit the establishment of tumours in 3 dimensional tissue culture assays. Moreover, if early IL-12Fc treatment only depends on this initial obstruction of tumour formation, a single bolus of IL-12Fc would be expected to cause the same suppression of tumour growth as early treatment. In contrast, the low number of tumour cells at the time of inoculation may be more controllable by the few tumour suppressive immune cells.

Previous studies suggested the importance of direct effects of IFNγ on the tumour cells, resulting in suppression of their proliferation (280) and induction of anti-angiogenic effects

(191,280). The B16F10 cells employed in our experiments, however, always entailed the same responsiveness to IFNγ. Thus, the failure of the late IL-12-mediated anti-tumour response in *Ifngr1*$^{-/-}$ mice suggests that direct IFNγ signalling on B16F10 cells is dispensable in our model. To exclude that autocrine positive feedback loops are necessary for the maintenance of sufficient IFNγ levels, one could further determine the concentration of IFNγ tumours from *Ifngr1*$^{-/-}$ and WT mice.

For the study of early anti-tumour response, it is essential to bear in mind that the late IFNγ-dependent anti-tumour effect occurs in addition to the IFNγ-independent early response. Thereby, the late tumour suppression may mask the failure of early treatment in the genetically modified mouse models used in this study. Thus, the study of early and late tumour suppression in different knock out strains was predominantly performed simultaneously. The quantification of tumour sizes on day 7 of tumour growth, where early treatment already shows its tumour suppressive effect, however, could be biased due to small lesions and resulting inaccuracy of the measurements. To avoid confusion between the effects of early and late treatment, one could block the late IL-12Fc-induced anti tumour response by administration of an anti-IFNγ antibody to tumour-bearing mice.

In summary, our results support the fact that the mechanism of tumour rejection upon IL-12 administration is highly dependent on the timing of the treatment regimen.

»11 Sources of IFNγ upon late treatment

In late IL-12Fc-treated tumours, IFNγ was solely secreted by lymphoid cells. This contrasts previous reports showing that also myeloid cells can produce IFNγ upon IL-12 stimulation, although these findings are controversial (281). For instance, a subset of CD8α^{+} lymphoid DCs was observed to contribute strongly to the IFNγ secretion in response to IL-12 and listeria monocygotes stimulation in NK cell depleted *Rag2*$^{-/-}$ mice (282). In fact, serum levels of IFNγ were observed to be comparable to those of WT mice, whereas sera of *Rag2*$^{-/-}$*Il2gc*$^{-/-}$ mice lacked IFNγ (282). Whether such compensation also takes place in the tumours of anti-NK1.1 treated *Rag1*$^{-/-}$ mice in our study remains to be established. This sustained IFNγ secretion may explain the unaffected anti-tumour response in those mice. Particularly, the contribution of ILCs to intratumoural IFNγ production in the absence of NK and adaptive immune cells needs to be further characterised. Given the abundance of NK cell markers expressed on the surface of ILCs with group 1 function, these cells may also be targeted by anti-NK1.1 treatment. Indeed, preliminary data suggests a loss of splenic RORγt^{fm+} ILCs upon

both anti-NK1.1 and anti-asialo GM1 treatment (Data not shown). These experiments need to be repeated with focus on ILC populations in the tumour tissue. Moreover, IL-15 was suggested to be essential for the generation and/or maintenance of RORγt^{fm+} cells with ILC1 function. Thus, such cells would also lack in *Il15ra*$^{-/-}$ mice, in which the anti-tumour response is maintained.

By employing *Rorc*$^{-/-}$*Rag1*$^{-/-}$ mice, RORγt-dependent ILCs could be excluded as mediators of the late IL-12Fc-induced anti-tumour response. In these animals, classical ILC1s are, however, still present (50). Furthermore, the absence of RORγt-dependent ILCs was only confirmed in the small intestine of *Rorc*$^{-/-}$*Rag1*$^{-/-}$ mice. Thus, we cannot exclude that the splenic RORγt^{fm+} ILCs detected in *Rorc*$^{fm+}$*Rag1*$^{-/-}$ fate map animals truly depend on RORγt expression and are absent from *Rorc*$^{-/-}$*Rag1*$^{-/-}$ mice.

Similar to the study of Park and colleagues (196) the late IL-12Fc-mediated anti-tumour response of s.c. B16F10 melanomas was observed to fail in *Rag2*$^{-/-}$*Il2r*$^{-/-}$ mice, but was independent T, B or NK cells. The findings suggest that these lymphocyte populations are dispensable for IFNγ production. Furthermore, these data also indicate that the depleted lymphocytes are not required to respond to IFNγ in order for the IL-12Fc-mediated anti-tumour immunity to occur. The sustained tumour suppression in NK cell depleted *Rag1*$^{-/-}$ mice implies that also IFNγ stimulated cytotoxic effector functions are dispensable in this system. Although phenotypic and functional similarities between ILCs, NK and T cells have been described, previous reports have failed to demonstrate cytotoxic activity of ILCs (222-224,234). In order to further exclude the autocrine stimulation of lymphocytes by IFNγ to activate cytotoxicity, we aim to investigate the anti-tumour response upon late IL-12Fc treatment in perforin-deficient animals.

» 12 IFNγ controls the infiltration of leukocytes to the tumour

The treatment with IL-12Fc led to the accumulation of immune cells at the tumour site, as previously observed (198). This effect was highly dependent of IFNγ on day 10 of tumour growth upon late IL-12Fc treatment, as it was not observed in *Ifngr1*$^{-/-}$ mice. Thus, the presence of intratumoural leukocytes correlated with the suppression of tumour growth. This suggested that immune responses towards the tumour tissue were important and that the IFNγ responsive population may be of hematopoietic origin. The abundance of leukocytes in treated tumours could reflect on a facilitated migration of effector cells to the tumour tissue due to a local inflammatory response caused by IL-12Fc-induced IFNγ. Similar to previous

results (198), the elevated number of intratumoural leukocytes was shown to result from an increase of both lymphocytes and myeloid cells (Data not shown). IL-12Fc-mediated alterations of the tumour vasculature are therefore likely to support the migration of inflammatory cells to the tumour. Interestingly, the numbers of $CD45^+$ cells was more comparable in IL-12Fc treated and control tumours at later stages of tumour progression. The usage of a density gradient to enrich leukocytes from large tumours on day 19 may have influenced these results. This finding could also reflect the abundance of tumour cells in the exponentially growing lesions at late stages.

»13 Monocytes and their progeny are dispensable for late IL-12-mediated tumour rejection

The fraction of myeloid cells, among tumour infiltrating leukocytes, was observed to be particularly large in IL-12Fc treated tumours on day 10 of growth. The many stimulatory effects of IFNγ on this immune cell compartment described in the literature (174) supported the hypothesis that the IFNγ responsive tumour suppressing cell type may be of myeloid origin. Similar to previous studies, we found the majority of the intratumoural myeloid population was composed of monocytes and monocyte-derived cells that are dependent on CCR2 signalling (96-98). IL-12Fc treatment induced an increase of $MHCII^+Ly6C^{high}Ly6G^-$ cells within tumours. This indicated monocyte differentiation to macrophages or monocyte-derived inflammatory DCs (35). In addition, the co-expression of CD11c by the majority of $MHCII^+$ cells was indicative of this differentiation. Although the accumulation of MHC class II expressing cells suggests increased antigen presentation within IL-12Fc treated tumours, the anti-tumour response was shown to be independent of T cell responses. Thus, we interpret the elevated numbers of $MHCII^+$ cells as a general activation and differentiation of the monocytic compartment to exert pro-inflammatory functions. A small fraction of the $Ly6C^{high}Ly6G^-MHCII^+CD11c^+$ monocyte-derived cells expressed iNOS upon IL-12 stimulation. These cells are reminiscent of monocyte-derived TNF and iNOS producing (Tip)-DCs, described in infectious models (35) Therefore, it may be of interest to screen for TNF secretion within the tumour tissue. Although previous reports have related iNOS activity with tumour promoting functions of myeloid cells (94), in our study, iNOS expression correlated with the suppression of tumour growth. iNOS production was completely dependent on IFNγ signalling, which is supported by the literature (174). Since iNOS expression was confined to

the monocytic compartment, *Ccr2*$^{-/-}$ mice should be examined for the potential lack of iNOS^{+} cells.

Our experiments in *Ccr2*$^{-/-}$ mice excluded a role of monocyte-derived cells in IL-12Fc-mediated tumour suppression. Notably, an increased influx of Ly6G^{high} granulocytes seemed to compensate for the loss of monocytes. The LysM specific depletion of IFNγ signalling did not lead to impaired tumour suppression by IL-12Fc. The pSTAT1 analysis of blood leukocytes suggests that the IFNγR had been depleted on granulocytes. However, we still need to determine, which specific myeloid cell subsets lost IFNγ signalling in tumours of *LysM*-Cre^{+}*Ifngr*$^{fl/fl}$ mice. Thus, an involvement of myeloid cells in the IL-12Fc-induced anti-tumour response cannot be excluded. This notion is further supported by the presence of residual F4/80 expressing cells within tumours of *Ccr2*$^{-/-}$ mice. CCR2-independent tumour-associated macrophages may potentially be directly targeted by the injection of an anti-CSFR1 depleting antibody into tumour bearing mice, as demonstrated previously (283).

The function of DC subsets has previously been implied to be essential for the rejection of tumours upon IL-12Fc administration (196). DCs were, however, never excluded to be part of the tumour suppressive mechanism upon late IL-12Fc treatment in our study. These cells should be targeted by using *CD11c*-Cre *Ifngr*$^{fl/fl}$ mice, in which DCs would be unresponsive to IFNγ. Most of the DCs within tumours were, however, shown to be monocyte-derived and were therefore absent from *Ccr2*$^{-/-}$ mice. A small proportion of CD11b^{-} DCs were identified within the tumour tissue, but absolute numbers were generally low and did not increase upon IL-12Fc administration. Thus, it seems unlikely that DCs play a major role in the rejection of IL-12Fc treated tumours.

In conclusion it is not entirely clear, whether the IFNγ-responsive cell type, mediating the IL-12Fc-induced tumour rejection, can be found within the hematopoietic cell pool. For this reason, we plan to compare the effect of IL-12Fc on tumour growth in bone marrow chimeras, transferring *Ifngr1*$^{-/-}$ bone marrow to WT animals and *vice versa*. Thereby, we may be able to distinguish effects of IFNγ on hematopoietic cells and stromal populations of the B16F10 tumours.

»14 The tumour endothelium is not required to respond to IFNγ for late tumour rejection

We hypothesised that stromal cells may be involved, as various leukocyte populations were excluded to be essential for the IL-12Fc-mediated tumour suppression. The IFNγ-induced anti-angiogenic effect has been considered to be an important pillar of the tumour suppression elicited by IL-12 (183,274). Both direct and indirect effects of IFNγ have been suggested to impact the tumour endothelium (188). Our data confirms that IFNγ induces alterations of the vasculature in IL-12Fc treated tumours. This was illustrated by an up-regulation of adhesion molecules and decreased endothelial cell numbers. The data obtained by flow cytometry was further supported by immunohistochemical analysis of tumour sections. Similar to a previous report (284) our histological analysis indicated that particularly the microvasculature was reduced in IL-12Fc treated tumours. Examination of tumours from *VEC*-Cre^{+}*Ifngr*$^{fl/fl}$ mice suggested that the up-regulation of adhesion molecule expression and the reduction in endothelial cell number depended on direct IFNγ signalling on the tumour vessels. Due to expression of VE-Cadherin in hematopoietic stem cells (275,278) we also expect a proportion of mature leukocytes to be targeted in the *VEC*-Cre^{+}*Ifngr*$^{fl/fl}$ mice. Thus, modelling the anti-tumour response upon IL-12Fc administration in *VEC*-Cre^{+}*Ifngr*$^{fl/fl}$ mice may have limitations in distinguishing direct from indirect effects on the endothelium.

The data obtained in the *VEC*-Cre^{+}*Ifngr*$^{fl/fl}$ mice disconnects the observed effects of IL-12Fc treatment on the endothelium from effects on tumour growth. Thus, the anti-angiogenic effect in our model did not cause the tumour suppression, which is opposing a multitude of previous reports (183,186,274). In comparison to *Ifngr1*$^{-/-}$ mice we could still detect significant changes of ICAM-1 and VCAM-1 expression in *VEC*-Cre^{+}*Ifngr*$^{fl/fl}$ mice. The up-regulation was, however, not as pronounced as seen in Cre^{-}*Ifngr*$^{fl/fl}$ littermates and may be explained by an incomplete targeting of the endothelial cells in this system. We intend to investigate STAT1 phosphorylation in endothelial cells, in order to estimate the frequency of endothelial cells that have lost IFNγ responsiveness in *VEC*-Cre^{+}*Ifngr*$^{fl/fl}$ mice.

Interestingly, adhesion molecule expression did not correlate with the accumulation of immune cells in the tumour tissue at late stages of growth. The extensive necrosis observed in large B16F10 tumours and structural damage to the tumour vessels may support the influx of immune cells independent of trans endothelial migration. In contrast, IFNγ was shown to regulate the infiltration of leukocytes to the tumour at early stages (day 10) of tumour suppression. A characterisation of immune cells in day 10 tumours of *VEC*-Cre^{+}*Ifngr*$^{fl/fl}$ animals may clarify if endothelial alterations cause the infiltration of leukocytes.

Beyond the inhibition of endothelial cell growth and increase of adhesion molecules on tumour vessels, IL-12Fc treatment may also influence the vessel permeability or perfusion. A decrease in vessel perfusion upon IL-12 treatment was for instance demonstrated by Doppler ultrasound examination of murine tumours (285). These parameters of the tumour blood supply were not assessed in our studies. Moreover, indirect effects of IFNγ on the endothelium cannot be excluded to be important for the IL-12Fc-induced anti-tumour response. Thus, the impact of IL-12Fc-induced IFNγ on tumour vessels may still play a role in the rejection of late IL-12Fc treated tumours.

»15 Concluding remarks 1

The mechanism, by which IL-12Fc rejects solid B16F10 tumours upon late treatment, remains elusive. Similar to previous reports, IFNγ was attributed a central role in the process of tumour suppression (183,184). In regard to the clinical application, this finding is disappointing, as tumour regression and the occurrence of side effects have been linked to IFNγ signalling. While we have excluded several pathways, by which IFNγ was thought to affect tumour growth, we failed to show which cell type respond to IFNγ stimulation and causes the anti-tumour effect upon late IL-12Fc treatment. The tumour microenvironment, however, contains additional cell subsets, which could be the target of IFNγ. For instance, tumour cells induce the proliferation of fibroblasts and myofibroblasts, which contribute to the tumour structure by producing components of the ECM (4). The involvement of such cells is unlikely, as B16F10 tumours are known for their paucity in tumour-associated fibroblasts (personal communication with Kristian Pietras, Lund University). Several distinct pathways may contribute to the anti-tumour effect of IL-12Fc stimulated IFNγ. In this case, the blockade of one mechanism would not affect tumour growth. Thus, the experimental abrogation of multiple IFNγ-induced pathways would be required and it is difficult to speculate which combination would succeed.

»16 Splenic RORγt^{fm+} ILCs are sufficient to induce early anti-tumour responses

The group of ILC3s have been discovered to harbour distinct subtypes, which exert remarkably diverse functions (50). Our group described the essential role of RORγt-

dependent ILCs in the IL-12Fc-mediated anti-tumour response (198). At this time, the knowledge about different ILC3 subtypes was still limited. Thus, further characterisation of the tumour suppressive cell type was required to understand, which subsets of ILCs were capable of eliciting this response. We found distinct subpopulations colonising spleen and small intestines of the gut, differing in terms of phenotype and function. Most prominently, RORγt^{fm+} ILCs derived from those two organs differed regarding their tumour suppressive potential upon IL-12Fc treatment. While splenic RORγt^{fm+} ILCs induced the rejection of s.c. tumours, ILCs from the gut did not influence tumour growth. Whether this observation solely relies in the difference in responsiveness to IL-12Fc remains to be established. In order to study the sensitivity of ILC to IL-12Fc activation, we determined transcriptional levels of the β2 subunit of the IL-12R, which was found to be elevated in splenic compared to gut ILCs. How this difference affects the biological stimulation with IL-12Fc is not entirely clear. A larger proportion of splenic RORγt^{fm+} ILCs secreted IFNγ compared to gut ILCs, further indicating elevated responsiveness to IL-12Fc. IFNγ was, however, shown not to be involved in the rejection of early treated tumours and thus does not explain the differences in anti-tumour activity.

Splenic RORγt^{fm+} ILCs appear similar to conventional NK cells, as they also express surface markers and transcription factors related to NK cells. Moreover, these ILCs show a paucity of ILC characteristic markers. In fact, distinction of splenic RORγt^{fm+} ILCs and NK cells was only achieved by the ILCs fate map positivity for RORγt. It is questionable whether this feature is sufficient to classify the RORγt^{fm+} population as an ILC3 subset and to segregate them from conventional NK cells. This separation is particularly problematic as splenic RORγt^{fm+} cells down-regulated the expression of RORγt. Thus, it is uncertain, whether RORγt is critical for the development and function of the splenic subset or if RORγt^{fm+} expression rather represents a stochastic event in such cells. The RORγt^{fm+} signal may be caused by leakiness of Cre expression in *Rorc*-Cre mice. In contrast, the difference in anti-tumour activity between RORγt^{fm+} ILCs and NK cells clearly shows that these cells are functionally distinct. While RORγt^{fm+} ILCs suppressed tumour growth of B16F10-IL-12Fc tumours, fm^{-} splenic NK failed to do so. Interestingly, all RORγt^{fm+} ILCs in the tumours were found to express Thy-1 to the same extent as gut ILC3s and thereby segregate from their splenic counterpart. There is still no understanding of the origin of the tumour suppressive ILCs under more physiological conditions of IL-12Fc administration. These ILCs may migrate to the tumour from a distant organ or may be resident within the subcutis. Thus, Thy-1 expression could be induced on tumour infiltrating RORγt^{fm+} ILCs or characterise a specific population of resident cells. Therefore, it would be intriguing to evaluate, whether co-injected ILCs form the spleen

upregulate Thy-1 expression in the tumour tissue. Moreover, the uniform expression of Thy-1 creates the opportunity of experimentally depleting intratumoural RORγt^{fm+} ILCs by injection of an anti-Thy-1 antibody. This has previously been proven to be a successful approach for the elimination of ILCs in the gut (258).

Transition of ILC3s towards ILC1 function occurs upon the stimulation with IL-12 and involves the down-regulation of RORγt and induction of T-bet expression (240). As splenic RORγt^{fm+} ILCs were shown to lack RORγt and instead expressed T-bet, we assume that this population has adopted ILC1 characteristics. Whether these cells are more likely to accumulate in the spleen or if the organ microenvironment fosters ILC3 to ILC1 transition is not known. It is unclear why gut ILC3s cannot undergo this transition in the tumour, where they are exposed to IL-12Fc. As we assume that the tumour suppressive effects of ILCs occur during tumour establishment, gut ILC3s may not acquire ILC1 characteristics in time to exert this function. Thus, it would be interesting to induce gut ILC3s to attain ILC1 function by IL-12Fc stimulation *in vitro* to thereafter test their tumour suppressive capacity. The RORγt^{fm+} population in the spleen may also represent a distinct cellular subset compared to ILCs colonising the small intestines.

ILC1s have been shown to lack (286) or express (237) the transcription factor Eomes. A preliminary experiment suggested that the majority of RORγt^{fm+} ILCs from the spleen expressed Eomes, whereas this transcription factor was absent from most gut ILC3s. In that regard, splenic ILCs seem to be similar to the intraepithelial ILC1s described by Fuchs and colleagues (237). The Eomes^{+} fraction among the NKp46^{+} cells in the spleen highly depended on IL-15 signalling. The anti-tumour response upon early IL-12Fc treatment was, however, not compromised in *Il15ra*$^{-/-}$ mice. These results suggest that the anti-tumour response persists in absence of RORγt^{fm+}Eomes^{+} splenic ILCs. The potential existence of other cell populations with similar anti-tumour properties within these mice may explain this observation. The finding of Eomes^{-}NKp46^{+} cells suggests that the spleen contains an Eomes^{-} ILC1 subset. In contrast to the Eomes expressing fraction, this population was not markedly reduced in the absence of IL-15 signalling and could potentially exert the tumour suppressive function upon IL-12Fc activation. The hypothesis that RORγt^{fm+} ILCs may not be the only cell type exerting anti-tumour function was further supported by the persistent tumour suppression in *Rorc*$^{-/-}$*Rag1*$^{-/-}$ mice upon IL-12Fc treatment. Together, these data suggest that RORγt^{fm+} ILCs are sufficient, but not necessary for the anti-tumour response upon early IL-12Fc treatment. Conversely, this could indicate that splenic RORγt^{fm+} ILCs are not dependent on the expression of RORγt, and are thus still present in mice, which lack this

transcription factor. As the distinction of RORγt^{fm+} ILCs and NK cells is only possible in *Rorc*$^{fm+}$ mice, we could not confirm the absence of splenic ILCs in *Rorc*$^{-/-}$*Rag1*$^{-/-}$ mice.

The transfer of *Rorc*$^{-/-}$*Rag1*$^{-/-}$ splenocytes additionally supports that RORγt^{fm+} ILCs are not required during the early IL-12Fc-induced anti-tumour response. Moreover, this experiment contrasts the failure of the anti-tumour response upon transfer of *Rorc*$^{-/-}$ splenocytes performed previously (198). To what extend the absence of T cells in *Rorc*$^{-/-}$*Rag1*$^{-/-}$ compared to *Rorc*$^{-/-}$ mice would explain these differences is unclear. A report by Hepworth et al. suggested that RORγt expressing ILCs can regulate T cell function. The absence of ILCs was shown to leave the T cells in a hyper responsive state, causing spontaneous intestinal inflammation (287). In *Rorc*$^{-/-}$ animals such pathogenic T cells may potentially influence tumour growth. We aim to explore this by a side-by-side comparison of tumours supplied with *Rorc*$^{-/-}$*Rag1*$^{-/-}$ or *Rorc*$^{-/-}$ splenocytes.

» 17 Concluding remarks 2

Although IL-12Fc responsive RORγt^{fm+} ILCs were confirmed to be sufficient in suppressing tumour growth, they do not seem to be necessary, as demonstrated by the intact anti-tumour response in their absence. This function is therefore unlikely to be exclusive for RORγt^{fm+} ILCs, but is rather supported by multiple cells types. The nature of such populations has not yet been determined. It is possible that RORγt^{-}independent ILC subsets could bear anti-tumour capacity. The question of how such ILCs suppress tumour growth remains to be answered. Interference with tumour tissue formation would be one potential mechanism. Due to the low number of RORγt^{fm+} ILCs observed in tumours, further stimulation of other cell types is likely required during tumour rejection.

The action of RORγt^{fm+} ILCs seems to be restricted to the very early phases of tumour suppression. This certainly puts the clinical relevance of the early treatment and the role of ILCs into perspective, as cancer patients usually present themselves at late stages of tumour growth. The strong tumour suppressive capacity may, however, present a novel function of ILC subsets and thus further contributes to the characterisation of those cells. Moreover, unravelling the anti-tumour function of ILCs could lead to better understanding of the various mechanisms, by which the immune system controls cancer development and growth.

VIII Appendix: T Cell Contamination in Flow Cytometry Gating Approaches for Analysis of Innate Lymphoid Cells

»18 Results and Discussion

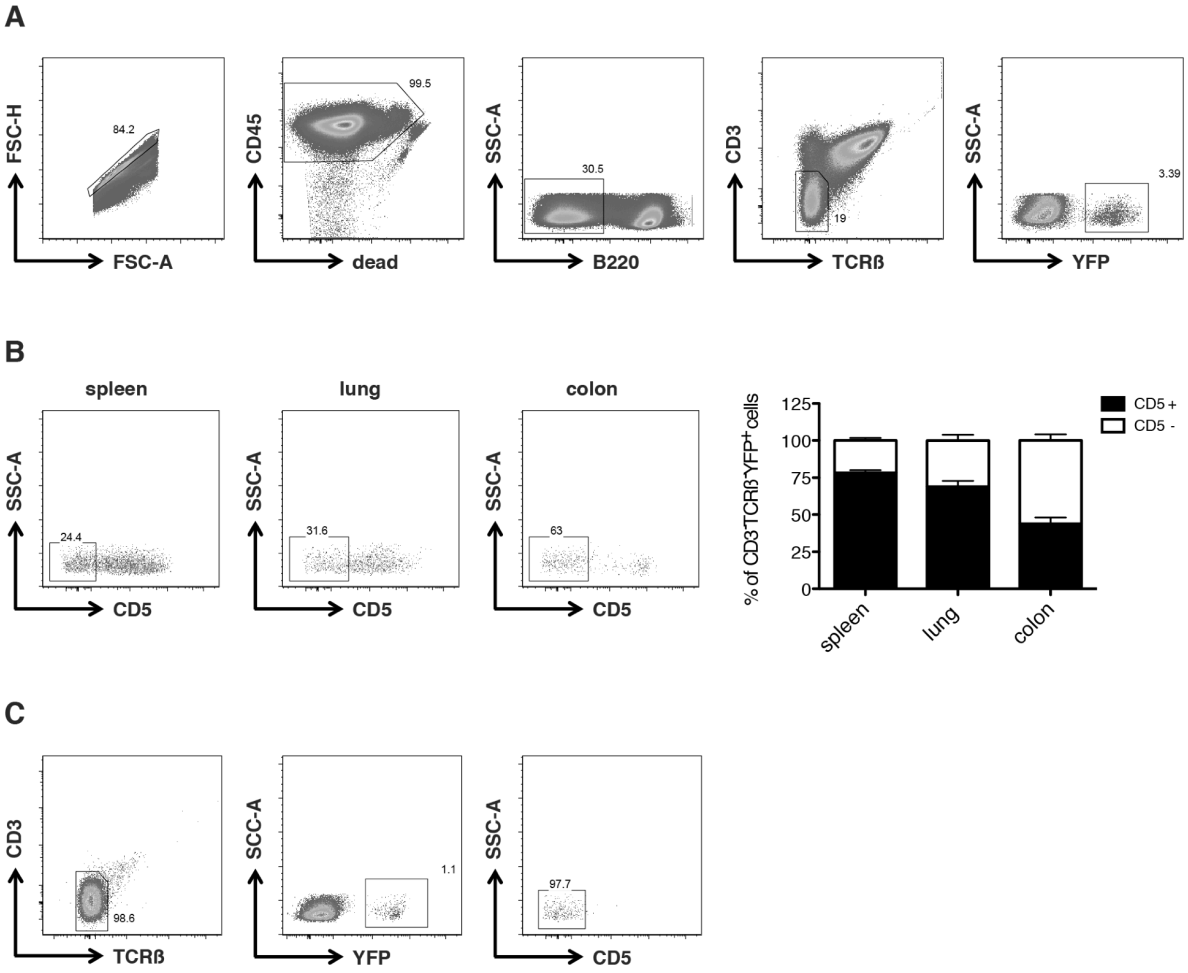
»18.1 CD5+ fraction remains in the ILC3 gate after exclusion of T cells using CD3 and TCRβ

Besides its importance for ILC3s, RORγt is also involved in the development of T cells, mainly T_H17 and γδ T cells (217,288). However, most ILC3 and T cell subsets will subsequently down-regulate the expression of RORγt (239). By utilising *Rorc*$^{fm+}$ mice, the fate of RORγt^{fm+} positive RORγt-dependent cells was mapped (199).

Using leukocytes isolated both from lymphoid (spleen) and non- lymphoid organs (colon and lung), we compared different gating approaches for the efficiency in separating ILC3s and T cells. The gating strategy involved exclusion of doublets, dead cells and B220 expressing events from CD45$^+$ leukocytes. In order to exclude T cells, CD3 and TCRβ positive events were negatively selected followed by gating on RORγt^{fm+} cells (Figure 1a). Similar gating approaches are used to characterize both murine and human ILCs but are often not depicted in any figure or involve gating out multiple lineage markers in one fluorescent channel (241,242,289,290). However, upon further scrutiny of the assumed ILC3s, we discovered a significant percentage of these cells to stain positive for CD5, a molecule described to inhibit the T and B cell receptor signalling and was historically used as a pan T cell marker. Whereas in the colon this frequency was relatively low with 43.9% CD5$^+$ cells within the ILC3 gate, in spleen and lung it reached 78.2% and 68.9%, respectively (Figure 1b). Strikingly, in *Rorc*$^{fm+}$*Rag1*$^{-/-}$ mice no expression of CD5 was detected, suggesting that the CD5$^+$ cells obtained from *Rorc*$^{fm+}$ mice belong to the T cell lineage (Figure 1c). Notably, as CD5 is upregulated upon TCR engagement, it is highly expressed in activated TCR^{low} and CD3^{low} T cells, which will appear close to the ILC gate (291).

Figure 1. Flow cytometric analysis of ILC3s using *RORc*$^{fm+}$ fate map mice. (A) Gating strategy for ILC3s in *Rorc*$^{fm+}$ mice: after exclusion of doublets, live CD45$^+$B220$^-$CD3$^-$TCRβ$^-$ RORγt^{fm+} cells were selected. (B) The population gated in panel A contains a significant fraction of CD5$^+$ cells both in spleen, lung and colon. (C) In *Rorc*$^{fm+}$*Rag1*$^{-/-}$ mice all RORγt^{fm+} CD45$^+$ live cells stain negative for CD3, TCRβ and also CD5. Representative plots for n = 3 (mean ± S.E.M).

Figure 1



»18.2 CD5+ cells remaining within the CD32 TCRb2 ILC3 gate consist of αβ T cells

To determine the nature of the CD5 expressing CD3$^-$TCRβ$^-$ cells, this population was purified by flow cytometric cell sorting from the spleen (Figure 1a and b). The mRNA levels for the constant region of the T cell receptor α and δ chain (TRAC and TRDC) were compared to that of CD5$^-$ ILC3s, αβ and γδ T cells as well as monocytes. As a genetic control we used ILC3s derived from *Rorc*$^{fm+}$*Rag1*$^{-/-}$ mice. Whereas CD3$^-$TCRβ$^-$CD5$^-$ ILC3s showed minor expression of both TRAC and TRDC (probably reflecting inevitable contamination from the sort purification), purified αβ and γδ T cell populations as expected transcribed high levels of TRAC or TRDC, respectively (Figure 2). Strikingly, when analyzing TRAC and TRDC expression levels in CD3$^-$TCRβ$^-$CD5$^+$ cells we detected high levels of TRAC, suggesting that this population indeed consisted mainly of αβ T cells.

Figure 2

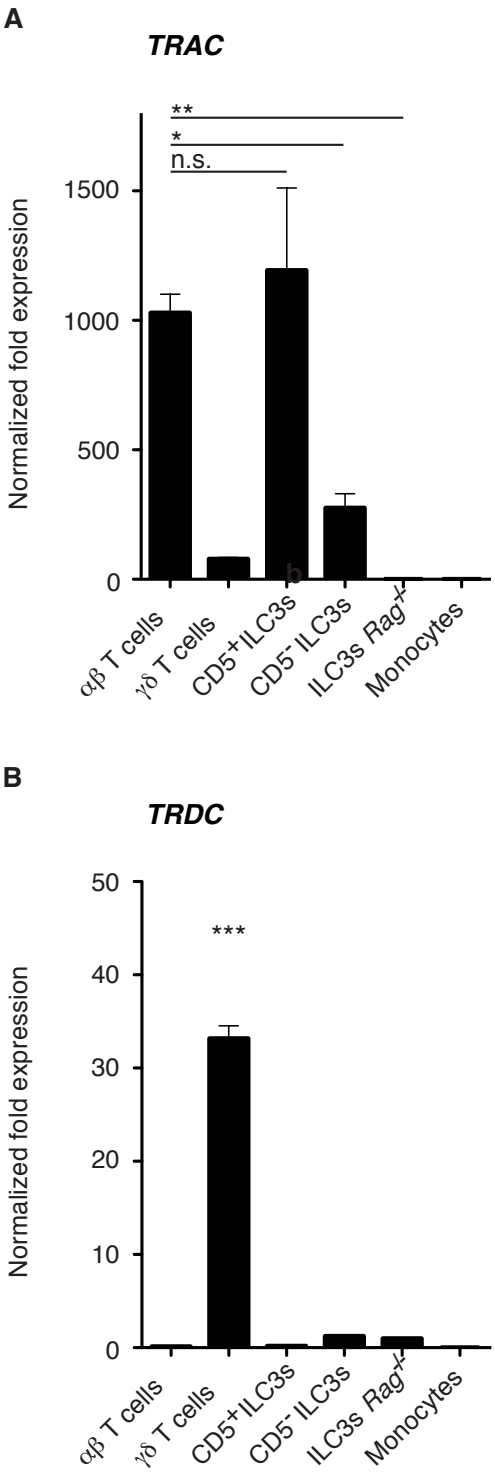


Figure 2. CD5^{+} cells within the ILC3 gate express high levels of TRAC. Normalized fold expression for TRAC (A) and TRDC (B) comparing CD5^{+} and CD5^{-} cells within the ILC3 gate ("CD5^{+}ILC3s", pregated on CD45^{+}B220^{-}CD3^{-}TCRβ^{-} RORγt^{fm+} cells), αβ and γδ T cells, monocytes (Mono) purified from *Rorc*$^{fm+}$ mice and ILC3s derived from *Rorc*$^{fm+}$*Rag1*$^{-/-}$ mice (ILC3s *Rag*$^{-/-}$). Results are presented relative to those obtained from ILC3s of *Rorc*$^{fm+}$*Rag1*$^{-/-}$ mice (mean ± S.E.M).

»19 Concluding remarks

We conclude that gating on lineage negative cells only using CD3 is not sufficient to firmly exclude αβ T cells from further analysis. This problem becomes particularly evident, when a CD3 containing ‘‘dump channel’’ is employed, where cells expressing lineage markers at different intensities are gated out in a single fluorescence channel. Furthermore, the scarcity of ILCs together with the high number of T cells, which depending on their activation status might express different levels of CD3, seems to cause limits for a separation based on one T cell marker. Thus, results obtained from contaminated ILC populations will be biased and T cells will contribute to the detected cytokine levels. This may not only produce artefacts but also mislead the characterization of ILC subsets according to the nomenclature based on cytokine and transcription factor expression.

Given that within the past years a strong association between ILC and T cell effector function has been proposed, we suggest that a thorough separation between these two populations is essential. Hence, lineage negative gating should not only be performed using CD3 only, but also CD5, preferably in a separate fluorescent channel.

Materials and Methods

» 19.1 Mice

C57BL/6 mice were purchased from Janvier. *Rag1*$^{-/-}$, *Rag2*$^{-/-}$*Il2rg*$^{-/-}$, *Ifngr1r*$^{-/-}$, *Rorc*^{GFP}, *Il12rb2*$^{-/-}$ and *LysM*-Cre mice were acquired from Jackson Laboratories. S. Bulfone-Paus (University of Manchester) provided us with *Il15ra*$^{-/-}$ mice. VE-Cadherin-Cre mice were kindly contributed by C. Halin Winter (ETH, Zurich). W. Müller supplied the *Ifngr*$^{fl/fl}$ mice. *Rorc*-Cre x *ROSA*26-stop$^{fl/fl}$-eYFP mice were kindly provided by A. Diefenbach (Freiburg University, Germany). All mice were kept under specific pathogen free conditions and treated according to animal guidelines by the Swiss Cantonal Veterinary Office (License 147/2012, Zurich, Switzerland).

» 19.2 Inoculation of tumour cells, growth monitoring and animal handling

Cells were detached using trypsin and washed with PBS. After centrifugation at 400g for 5 min (conditions used for wash steps if not mentioned otherwise) cells were filtered through a 70mm mesh and diluted in PBS to the concentration of 2 x 10^6 cells/ml. The cell concentration was determined by using a Neubauer counting chamber. 2 x 10^5 B16F10 cells were injected s.c. into the left flank of each animal under isoflurane anaesthesia. For the transfer experiments B16F10-IL-12Fc cells were prepared as described above and 2x10^5 cells were mixed and co-injected with either 10'000 FACS purified leukocyte populations or 2x10^5 splenocytes per mouse. Tumour growth was monitored by perpendicular measurement of the diameter and calculation of the tumour area in mm^2 from day 7 of tumour growth. 10ml of IL-12Fc (200ng per injection) or PBS were injected s.c. before the development of a palpable lesion or i.t. after tumour formation and treatment was commenced on day 0 (early treatment) or day 7 (late treatment) in the experiment. Both determination of tumour size and injection of IL-12Fc or PBS took place three times a week. A tumour diameter of ≥ 20mm, ulceration of the lesion or body weight loss of ≥ 15% were the main withdrawal criteria for individual mice. Depending on the tumour growth in different mouse strains the experiments were terminated between day18 and 22.

For the depletion of NK cells in *Rag1*$^{-/-}$ mice we used the 200mg anti-NK1.1 (PK136, BioXCell) per mouse and injection. Treatment was initiated 5 days prior to tumour cell inoculation and continued every second day over the course of the experiment. NK cell depletion was confirmed in the blood of the mice using flow cytometry before B16F10 injection and thereafter weekly.

» 19.3 DNA isolation and genotyping

DNA was isolated from either ear or toe tip biopsies, which were digested using 400 ml of tail lysis buffer containing 8ml proteinase K for 2 h at 55°C. After centrifugation for 10 min at 13.000 rpm the supernatant was mixed with 400ml of isopropanol to precipitate the DNA and centrifuged for 10 min at 13.000 rpm and 4°C. Supernatants were removed, the pellet was washed with 100 µl of ice cold 70% ethanol, centrifuged for 5 minutes at 13.000 rpm and supernatants were discarded. Pellets were air-dried for 10-15 min, resuspended in 100 µl of TE buffer and kept at 4°C.

The genotyping was performed using the following PCR protocols and the amplicons were analysed using a 1-2% agarose gel in TEA buffer.

RORc-Cre

Primer sequences:

TTA CCG GTC GAT GCA ACG AGT

TTC CAT GAG TGA ACG AAC CTG G

PCR reaction mixture per sample:

10X PCR buffer with MgCl2 (New England Biolabs):	2.5 µl
Primer each (10 µM)	0.5 µl
dNTPs (10 µM)	1.25 µl
Taq DNA Polymerase (New England Biolabs)	0.1 µl
Template (DNA)	2 µl
dH_2O	18.15 µl

Amplification:

1. 94°C 5 min
2. 94°C 30 sec
   58°C 1 min
   72°C 1 min repeat 35x
3. 72°C 10 min
4. keep at 12°C

Band analysed: 400bp

ROSA26-stop$^{fl/fl}$-eYFP

Primer sequences:

AAG ACC GCG AAG AGT TTG TC

AAA GTC GCT CTG AGT TGT TAT

GGA GCG GGA GAA ATG GAT ATG

PCR reaction mixture per sample:

10X PCR buffer with MgCl2 (New England Biolabs):	1.2 µl
Primer each (10 µM)	1.2 µl
dNTPs (10 µM)	0.24 µl
Taq DNA Polymerase (New England Biolabs)	0.06 µl
Template (DNA)	2 µl
dH_2O	4.9 µl

Amplification:

1. 94°C 3 min
2. 94°C 30 sec
   58°C 1 min
   72°C 1 min repeat 35x
3. 72°C 2 min
4. keep at 12°C

Bands analysed:

Transgene: 320 bp

WT: 600 bp

***Rag1*$^{-/-}$**

Primer sequences:

TGG ATG TGG AAT GTG TGC GAG

CCA GAC AAG TTT TTC ATC GT

GAG GTT CCG CTA CGA CTC TG

PCR reaction mixture per sample:

10X PCR buffer with MgCl2 (New England Biolabs):	3 µl
Primer 1 & 3 (10 µM)	0.3 µl
Primer 2 (10 µM)	0.15 µl
dNTPs (10 µM)	0.3 µl
Taq DNA Polymerase (New England Biolabs)	0.1 µl
Template (DNA)	2 µl
dH_2O	5.85 µl

Amplification:

1. 94°C 3 min

2. 94°C 30 sec
   58°C 45 sec
   72°C 30 sec repeat 35x
3. 72°C 2 min
4. keep at 12°C

Bands analysed:

Rag1$^{-/-}$: 530 bp

WT: 474 bp

***Rorc*^{GFP}**

Primer sequences:

CCC CCT GCC CAG AAA CAC T

GGA TGC CCC CAT TCA CTT ACT TCT

CGG ACA CGC TGA ACT TGT GG

PCR reaction mixture per sample:

10X PCR buffer with MgCl2 (New England Biolabs):	2 µl
Primer 1 & 3 (10 µM)	1 µl
dNTPs (10 µM)	1 µl
Taq DNA Polymerase (New England Biolabs)	0.5 µl
Template (DNA)	1 µl
dH_2O	12.5 µl

Amplification:

1. 94°C 3 min
2. 94°C 30 sec
   60°C 30 sec
   72°C 30 sec repeat 35x
3. 72°C 5 min
4. keep at 12°C

Bands analysed:

Rorc^{GFP}: 241 bp

WT: 174 bp

***VEC*-Cre (general Cre)**

Primer sequences:

Transgene	GCG GTC TGG CAG TAA AAA CTA TC
	GTG AAA CAG CAT TGC TGT CAC TT
Internal control	CTA GGC CAC AGA ATT GAA AGA TCT
	GTA GGT GGA AAT TCT AGC ATC ATC C

PCR reaction mixture per sample:

10X PCR buffer with MgCl2 (New England Biolabs):	1.2 µl
Primer each (10µM)	1.2 µl
dNTPs (10µM)	1.25 µl
Taq DNA Polymerase (New England Biolabs)	0.1µl
Template (DNA)	1 µl
dH_2O	4.65 µl

Amplification:

1. 94°C 3 min
2. 94°C 30 sec
   51°C 1 min
   72°C 1 min repeat 35x
3. 72°C 3 min
4. keep at 12°C

Bands analysed:

Transgene: 100bp

Control: 324 bp

***LysM*-Cre**

Primer sequences:

CTT GGG CTG CCA GAA TTT CTC

TTA CAG TCG GCC AGG CTG AC

CCC AGA AAT GCC AGA TTA CG

PCR reaction mixture per sample:

10X PCR buffer with MgCl2 (New England Biolabs):	2.5 µl
Primer each (10µM)	0.5 µl
dNTPs (10µM)	1.25 µl
Taq DNA Polymerase (New England Biolabs)	0.1µl
Template (DNA)	1 µl
dH_2O	18.9 µl

Amplification:

1. 94°C 3 min
2. 94°C 30 sec
   62°C 30 sec
   72°C 1 min repeat 35x
3. 72°C 3 min

4. keep at 12°C

Bands analysed:

LysM-Cre: 700 bp

WT: 350 bp

» 19.4 Production and purification of IL-12Fc

For the production of IL-12Fc the 293T cell line (human embryonic kidney cells) were transfected using co-precipitates of calcium phosphate and vector DNA (pCEP4-mIL-12IgG3). The transfection was performed according to standard protocols using 25µg of DNA per 15cm tissue culture plate. Cells were thereafter cultured in high glucose DMEM medium (PAN-Biotech), containing 10% fetal calve serum (FCS), 1% penicillin streptomycin, 1% L-glutamine and 1% sodium pyruvate (media containing these supplements will be referred to as complete media), which was substituted with 1% nutridoma SP (Roche). The supernatant was collected 2-3 days and 4-6 days after transfection depending on cell density in the culture dish. After centrifugation at 3520 g for 10 min the supernatant was sterile filtered through a 0.22mm mesh. IL-12Fc was then purified using a protein G column (1ml, HiTrap, GE Healthcare) and the ÄktaPrime purifier. The protein elution was performed using 0.1 M citric acid (pH 3.0) and the flow through was collected in 1M Tris pH 8.8. This step was followed by an over night dialysis of the eluate in PBS pH 7.4, and purified IL-12Fc was stored at -20°C. IL-12Fc concentration was measured by a BCA assay (Pierce™ BCA Protein assay kit, Thermo Scientific) and by p40 ELISA (OptEIA Il-12/23p40, BD Pharmingen). Protein purity was determined by SDS-PAGE followed by a silver staining and immunoblotting. IL-12Fc was detected with a rat anti-mouse IL-12p40 antibody (C17.8, BioExpress) and a goat anti-rat HRP coupled antibody (Jackson).

» 19.5 Cell lines and culture conditions

B16F10 cells were obtained from ATCC and propagated in complete DMEM medium. B16F10-IL-12Fc were generated by Johannes vom Berg and Maya Eisenring in our group as described in Eisenring et al. (198). These cells were cultured in complete DMEM medium containing 0.25 mg/ml hygromycin and 0.2 mg/ml zeocine. The MS1 cell line was kindly provided by M. Detmar (ETH, Zurich) and kept in complete DMEM medium. All cells were passaged every second day.

»19.6 *In vitro* assay using MS1 cells

MS1 cells were labelled using 1μM cell trace violet (Molecular Probes) working solution and a cell density of $1x10^6$ cells/ml. Thereafter 2000 MS1 cells were plated per well in a 98 well cell culture dish. 6'000-10'000 FACS purified lymphocyte populations were added with or without 5 ng/ml IL-12Fc. The anti-IFNγ antibody (R4-6A2; BioExpress) was utilised at a concentration of 10 ng/ml and 10 ng/ml IFNγ was added as a positive control. The MS1 cells were then cultured for 48 hours in RPMI (PAN-Biotech) complete. After removing the non-adherent lymphocytes the MS1 cells were incubated with 1/5 diluted trypsin for 3 min followed by mechanical disturbance to detach the cells. After a wash with PBS, the cells were stained for flow cytometric analysis.

»19.7 Isolation of leukocytes from tumour tissue

Mice were euthanized using rising concentrations of CO2 on day 5 or 10 of tumour growth or at the endpoint of the tumour experiment. Tumours were resected and separated from skin and adjacent subcutaneous tissue and weight of the lesion was determined. The tissue material was in general kept on ice or at 4°C if not mentioned otherwise. Tumours were cut into small pieces and processed in digestion medium (10ml/gram of tumour tissue) under continuous agitation at 37°C for 45 min. Digestion medium consisted complete RPMI containing collagenase IV (Sigma) 0.4mg/ml and DNAse (Roche) 100 μg/ml. The lysate was further disrupted using a syringe and a 19 gauge needle and was thereafter filtered through a 70 mm cell strainer and washed with PBS. At this stage tumours obtained on day 5 or 10 of tumour growth were then stained for flow cytometric analysis immediately or taken in culture for restimulation. The leucocytes in lesions resected at later time points were further enriched by a density gradient using 70% and 40% isotonic Percoll (1.124 g/ml, Biochrom) and spun at 1350g for 30 min at 18°C without brakes. The interphase was collected and filtered through a 70 mm mesh and washed with PBS before continuing with further procedures.

»19.8 Isolation of leukocytes from tumour tissue

The isolation of endothelial cells was adapted from the procedure described in van Beijnum et al. (292). Tumours were resected from the flank of euthanized mice as described above and weight of the lesion was determined. The tissue was always kept on ice and in EC isolation medium if not described otherwise. All the media used in this procedure are listed below and were used at 4°C. Tumours were disrupted by cutting and processed in a

digestion medium - 10ml/gram of tumour tissue - and kept agitating at 37°C for 60 min. After 30 min of digestion DNAse (Roche, final concentration: 100 μg/ml) was added to the mixture. To increase the yield of endothelial cells the digested tumours were then mixed with EC isolation medium (5ml per 10ml digestion medium) containing heparin-Na (B. Braun) to avoid clustering of endothelial cells in thrombi and filtered through a 100μm mesh. The filter was then rinsed with additional 5 ml of EC isolation medium and pelleted by centrifugation. After discarding the supernatant a density gradient was performed to enrich tumour endothelial cells. The pellet was resuspendend in 4ml (tumours below 0.5 g) or 10ml (tumours above 0.5 g) of an anti-coagulating Ficoll medium and was layered on top of 3 ml or 7.5ml of Ficoll-Paque PLUS (1.0771+/-0.001g/ml), respectively. After centrifugation at 400g for 20min at 18°C without brakes the interphase was collected and washed with EC isolation medium. A second wash with PBS was performed and the samples were stained for flow cytometric analysis.

EC medium	complete RPMI with 20% FCS
EC medium+	EC medium containing: 0.001% Heparin-Na 25000 I.E/5ml (B. Braun) 1% NEAA (Gibco) 1% Hepes (Gibco) 1% MEM Vitamins (Gibco)
Conditioned EC medium	Medium from cultured MS-1 cells; filtered through a 0.2 μm mesh and stored at -20°C
EC isolation medium	80% EC medium+ and 20% conditioned medium
Digestion medium	RPMI only containing: Collagenase VI (Sigma) 0.4mg/ml Dispase (Gibco) 2.4mg/ml
Ficoll medium	EC medium containing 1% sodium citrate (stock 2M)

» 19.9 Isolation of leukocytes from spleen

After excision spleens were mechanically disrupted using a syringe plunger or by cutting them into small pieces. For the purification of ILCs from spleens the samples were at this point additionally incubated with collagenase IV (0.4mg/ml, Sigma) diluted in RPMI complete for 20 min at 37°C to increase the yield of cells. The lysate was then filtered through a 70mm mesh and washed with PBS. After centrifugation the pellet was resuspended in 1ml red blood cell (RBC) lysis buffer and kept on ice for 10 min. Splenocytes were then washed and stained for flow cytometric analysis, FACS sort or directly transferred to the animals.

»19.10 Isolation of leukocytes from the lamina propria of the small intestines and colon

The gut was removed and divided into small intestines (below the stomach to the entrance into the ceacum) and colon (ceacum to rectum). Mesenteric fat tissue and remaining faeces were removed; the intestinal tube was cut open and dissected into smaller pieces. The samples were then washed three times in 10 ml hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} containing 2% FCS (CM), vortexing the samples for 20 min with each wash step. Gut pieces were then incubated in CM containing 1 mM Dithiothreitol (DTT) and 1.35 mM EDTA for 15 min at 37°C to remove remaining mucus and the epithelial layer. After an additional incubation in CM complemented with EDTA for 30 min at 37°C the samples were cut into small pieces. The digestion steps described so far were performed under continuous agitation. The gut tissue was then digested using 0.4 mg/ml collagenase IV (Sigma) diluted in HBSS with Ca^{2+} and Mg^{2+} containing 10% FCS for 45 min at 37°C. The samples were then homogenised using a syringe with an 18 gauge needle, filtered through a 70 mm cell strainer and used for subsequent procedures.

»19.11 Isolation of leukocytes from the lung

Lungs were mechanically disrupted using scissors and were thereafter digested in IMDM containing 1 mg/ml Collagenase D (Roche), 0.5 mg/ml DNAse (Sigma Aldrich), 25 mM HEPES and 2% FCS for 60 min at 37°C. The remaining tissue pieces were additionally homogenised using syringes and 18 gauge needles, filtered through a 70 mm cell strainer and washed with PBS. Red blood cells were removed using RBC lysis buffer for 10 min, samples were washed and stained for flow analysis.

»19.12 Flow cytometry

Before IFNγ staining, isolated cells were *in vitro* re-stimulated for 4 h at 37°C using complete RPMI supplemented with PMA (50 ng/ml), Ionomycin (500 ng/ml) and GolgiPlug (diluted 1:1000). pSTAT1 was stained after cell stimulation with IFNγ (50 ng/ml) for 30 min and was performed according to the protocol by BD using the perm buffer II (BD). For all other intracellular staining cells were fixed for one hour in either Cytofix/Cytoperm buffer (BD) for IFNγ and iNOS staining or in Foxp3 Fixation/Permeabilization buffer for Eomes and RORγt, according to the manufacturers protocols. Wash steps were then performed in a Perm/Wash buffer from BD or a homemade solution containing Saponin. Flourochrome-conjugated or

biotinylated antibodies were purchased from BD, BioLegend or eBioscience and incubated with the samples for 20 min on ice. EOMES and RORγt were stained at 4°C over night. A list of the most frequently used antibody clones can be found below. Aqua Live/Dead fixable staining reagent (Invitrogen) was used to exclude dead cells, and single events were gated on using FSC-A vs. FSC-H. Compensation was performed using single stained anti-rat/hamster compensation beads, stained with the fluorophores used in the experiment. The samples were acquired using a LSR II Fortessa (special order research product, BD) with four laser lines (405 nm, 488 nm, 561 nm and 640 nm). The data was analysed using the FlowJo V9.x software (Treestar). Absolute cell numbers were calculated using Accu check counting beads (BD). For cell sorting a FACS Aria III (BD) was used.

Antibody	Dilution	Clone
anti CD3 - AF700 / PE Cy7	1:100 / 1:400	17A2
anti CD4 - PE-CF594	1:400	GK1.5
anti CD5 - APC	1:800	53-7.3
anti CD8 - FITC	1:400	53-6.7
anti CD11b - APC Cy7 / BV 605	1:400	M1/70
anti CD11c - PE-Cy7	1:400	N418
anti CD31 - FITC / PerCP Cy5.5	1:200 / 1:400	390
anti CD45 - APC-Cy7 / PB	1:400 / 1:800	30-F11
anti CD49b - PE / BIO	1:100	DX5
anti CD54 (ICAM-1) - APC	1:400	3E2
anti CD90.2 (Thy-1.2) - PB / AF700	1:200	30-H12
anti CD106 (VCAM-1) - FITC	1:100	429
anti CD117 (c-Kit) - APC	1:100	2B8
anti CD127 (IL-7Rα) - PE / PE-Cy7	1:100	SB/199
anti Ly-6A/E (Sca-1) - PE Cy7	1:200	D7
anti Ly-6C - FITC	1:400	AL-21
anti Ly6G - AF647	1:400	1A8
anti NK1.1 - V450 / AF700	1:100	PK136
anti NKp46 - perCP-eFluor 710	1:100	29A1.4
anti F4/80 - Biotin	1:200	CI:A3-1
anti TCR β - perCP Cy5.5	1:400	H57-597

anti TCR γδ - PE-Cy7	1:400	GL3
anti I-A / I-E (MHCII) -PB / AF700	1:800	M5/114.15.2
anti IFNγ - PE Cy7	1:400	XMG1.2
anti IL17A-eFlour660 (APC)	1:200	17B7
anti T-bet - BV605	1:200	4B10
anti RORγt - BV421	1:800	Q31-378
anti Eomes - PE	1:100	Dan11mag
anti pSTAT1-PE (pY701)	1:10	4a
anti-NOS2 (iNOS) - PE	1:100	CXNFT

» 19.13 Immunofluorescence

The tumour was excised with the surrounding skin and connective tissue and was fixed in HOPE I (BioSciences) solution for 48-72 h at 4°C. Samples were then dehydrated in 100% Acetone containing 1:1000 HOPE II (BioSciences) for two hours followed by three incubations in 100% Acetone only, each for 2 h. The tumours were then put in paraffin at 55°C over night and thereafter embedded, cut into 10 µm sections and freed from paraffin using isopropanol and acetone. Slices were then incubated with blocking buffer (PBS with 3% bovine serum albumin (BSA), 5% FCS, 0.1% Triton) for 40 min and stained with the primary antibodies at 4°C over night. The samples were then incubated with secondary antibodies at room temperature for 2 h. After washing the samples with PBS slides were mounted using mounting medium DAPI (Dianova). Imaging was performed using an inverse fluorescence microscope IX81 (Olympus) and analysed using the ImageJ64 software.

Primary Antibody	Dilution	Clone
anti CD31	1:500	MEC 13.3 (rat)
anti CD54 (ICAM-1) - PE / APC	1:200	3E2 (hamster)
anti F4/80 - Biotin	1:100	CI:A3-1 (rat)

Secondary Antibody	Dilution
goat anti-rat IgG AF488	1:500

»19.14 RNA isolation and quantitative PCR

Purified cells were resuspended in 1 ml trizol (Invitrogen) and stored at -80°C if the isolation was not performed immediately. 200 ml of chlorophorm was added, mixed in gel lock tubes and centrifuged at 13'200 g for 20 min at 4°C. The upper aqueous phase was collected and mixed with an equal volume of ice cold 70% ethanol. Thereafter RNA was extracted using the PureLink RNA Micro kit (Invitrogen) according to the manufacturers protocol. An additional wash step was performed with 80% ethanol at the end of the procedure and RNA was eluted in 12 ml of pre-warmed RNAse free water. RNA concentration was estimated using a spectrophotometer (NanoDrop). Subsequent first-strand cDNA synthesis was performed using Superscript II reverse transcriptase (Invitrogen) and oligo (dt) primers (PeproTech) according to the Invitrogen protocol. Quantitative analysis was conducted using a SYBR Green master mix (Roche) using the following master mix:

6.25 µl SYBR-Mix (2x)
3.25 µl H2O
0.25 µl of each primer
2.5 µl cDNA

The reaction was measured by a C1000 Touch thermal cycler (BioRad). mRNA levels were determined by the cycle threshold values and normalised to the expression of the Pol2 gene. Primers were designed using the Roche Universal probe library:

IL-12Rb2:	TGT GGG GTG GAG ATC TCA GT forward
	TCT CCT TCC TGG ACA CAT GA reverse
IL-23R:	CCA AGT ATA TTG TGC ATG TGA AGA forward
	AGC TTG AGG CAA GAT ATT GTT GT reverse
TRAC:	ACAAGCTTCACCTGCCAA forward
	GCTTTTCTCAGTCAACGTGG reverse
TRDC:	CTACGACTGCTGTTTGCCA forward
	TAGTCTCCTCATGTCAGCCC reverse

»19.15 Buffers

Biopsy lysis buffer	50 ml 1M Tris/HCl pH 7.5 5 ml 0.5M EDTA (AppliChem) 5 ml 20% SDS 20 ml 5M NaCl 500 ml H2O bidest

Red blood cell lysis buffer	4.15 g NH_4Cl 0.55 g $KHCO_3$ 0.185 g EDTA 500 ml H_2O bidest sterile filtered
Homemade Perm/Wash Buffer	PBS 2% BSA 0.5% Saponin
TAE buffer (50x)	484 g Tris base 14.2 ml acetic acid 200 ml 0.5M EDTA (pH 8.0) adjust pH to 7.5 2000 ml H_2O dest
TE buffer	10mM Tris/HCl pH 7.5 1mM EDTA (2 ml per 1000 ml of buffer)

» 19.16 Statistical analysis

Statistical analysis was performed using Prism version 5.0a (GraphPad Software Inc.). Statistical significance was determined by unpaired two-tailed T-test for the comparison of two groups or by one-way ANOVA with Bonferroni post-test unless mentioned otherwise. In case of significant differences in variances between the groups T-tests were adjusted using Welsh's correction. A p value below 0.5 was considered statistically significant.

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